THE FUNCTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR-B IN THE HEART

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To my mother

To arrive where you are, to get from where you are not,
You must go by a way wherein there is no ecstasy.
In order to arrive at what you do not know
You must go by a way which is the way of ignorance.
In order to possess what you do not possess
You must go by the way of dispossession.
In order to arrive at what you are not
You must go through the way in which you are not.
And what you do not know is the only thing you know
And what you own is what you do not own
And where you are is where you are not.

- T.S. Eliot, "East Coker"

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ABBREVIATIONS

AAV	adeno-associated virus
AMPK	adenosine monophosphate-activated protein kinase
αSMA	smooth muscle α-actin
ATP	adenosine triphosphate
Bmx	bone marrow kinase in chromosome X
CD	cluster of differentiation
CMC	cardiomyocyte
CT	computed tomography
DAG	diacyglycerol
Dll	delta-like ligand
E	embryonic day
EC	endothelial cell
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FATP	fatty acid transport protein
FDR	false discovery rate
Flk	fetal liver kinase
Flt	fms-like tyrosine kinase
GSK-3β	glycogen synthase kinase-3β
HIF	hypoxia-inducible factor
Ig	immunoglobulin
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
K14	keratin-14
KDR	kinase insert domain receptor
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MCP	monocyte chemotactic protein
MHC	myosin heavy chain
MI	myocardial infarction
MMP	matrix-metalloproteinase
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
NFAT	nuclear factor of activated T-cells
NO	nitric oxide
NRP	neuropilin
PAI	plasminogen activator inhibitor
PDGF	platelet-derived growth factor
PECAM	platelet endothelial cell adhesion molecule
PET	positron emission tomography
PGC-1α	peroxisome proliferator-activated receptor-γ coactivator-1α
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PlGF	placenta growth factor
RECA	rat endothelial cell antigen
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SMC	smooth muscle cell
sVEGFR-1	soluble VEGFR-1
TG	transgenic
Tie	tyrosine kinase with immunoglobulin and EGF homology domains
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
WT	wildtype

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text with their assigned roman numerals:

I Kärpänen, T.*, **Bry, M.***, Ollila, H.M., Seppänen-Laakso, T., Liimatta, E., Leskinen, H., Kivelä, R., Helkamaa, T., Merentie, M., Jeltsch, M., Paavonen, K., Andersson, L.C., Mervaala, E., Hassinen, I.E., Ylä-Herttuala, S., Oresic, M., and Alitalo, K. Overexpression of vascular endothelial growth factor-B in mouse heart alters cardiac lipid metabolism and induces myocardial hypertrophy. *Circulation Research* 103(9):1018-26 (2008).

II **Bry, M.**, Kivelä, R.*, Holopainen, T.*, Anisimov, A., Tammela, T., Soronen, J., Silvola, J., Saraste, A., Jeltsch, M., Korpisalo, P., Carmeliet, P., Lemström, K.B., Shibuya, M., Ylä-Herttuala, S., Alhonen, L., Mervaala, E., Andersson, L.C., Knuuti, J., and Alitalo, K. Vascular endothelial growth factor-B acts as a coronary growth factor in transgenic rats without inducing angiogenesis, vascular leak, or inflammation. *Circulation* 122(17):1725-33 (2010).

III Kivelä, R., **Bry, M.**, Robciuc, M.R., Taavitsainen, M., Silvola, J., Saraste, A., Hulmi, J.J., Räsänen M., Anisimov, A., Eklund, L., Hellberg, S., Hlushchuk, R., Zhuang, Z.W., Simons, M., Djonov, V., Knuuti, J., Mervaala, E., and Alitalo, K. VEGF-B-induced vascular growth leads to metabolic reprogramming and ischemia resistance in the heart. *Submitted.*

*These authors contributed equally to the study.

ABSTRACT

Despite intensive efforts, vascular growth factors have not yet provided significant help in the treatment of cardiovascular disease. This is likely to change as we gain a better understanding of the underlying biology of these growth factors as well as of their regulation and functions. Members of the vascular endothelial growth factor (VEGF) family are major regulators of blood and lymphatic vessel development and growth. VEGF is essential for vasculogenesis and angiogenesis, whereas VEGF-C is required for lymphatic development. The functions of VEGF-B, one of the younger members of the VEGF family, have however remained largely enigmatic.

This study was undertaken in order to elucidate the role of VEGF-B in the regulation of myocardial and vascular function in the heart, a site of high endogenous expression, as well as its therapeutic potential. For this end, VEGF-B was first overexpressed in the mouse heart, before proceeding to a larger transgenic rat model better suited for studies of cardiovascular physiology. VEGF-B overexpression did not cause overt angiogenesis but led to an increase in the size of capillaries in the heart. Surprisingly, VEGF-B also increased the size of cardiomyocytes, resulting in myocardial hypertrophy. The transgenic animals had significantly lower blood pressure and heart rate than their wild type littermates, and the isolated transgenic mouse hearts seemed to perform better following short-term ischemia-reperfusion.

Strikingly, in addition to myocardial growth, in rats VEGF-B induced impressive growth of the epicardial coronary arteries and their myocardial branches, which was associated with protection from myocardial infarction *in vivo*. However, in skeletal muscle and in the skin, VEGF-B did not significantly induce blood vessel growth, indicating that the heart is a site for specific effects of VEGF-B. These findings indicate that VEGF-B can act as a growth factor for cardiac vessels, which could have significant potential for therapeutic applications in cardiac insufficiency and/or ischemia. Importantly, compared with VEGF and placenta growth factor (PlGF), VEGF-B induced very little vascular permeability or inflammation.

The roles of the VEGF-B receptors, VEGFR-1 and neuropilin (NRP)-1, were also investigated, and the tyrosine kinase domain of VEGF receptor-1 (VEGFR-1) was found to be required for the cardiac hypertrophy induced by VEGF-B. NRP-1, however, did not seem to be involved in these effects. Interestingly, VEGF–VEGFR-2 signaling played a role in the cardiac vessel growth induced by VEGF-B.

In contrast to a prevailing theory, VEGF-B did not increase fatty acid uptake in the heart in our models. Instead, VEGF-B seems to play a role in fine-tuning cardiac metabolism to meet energy demands during for example cell growth. Overall, VEGF-B has potential as a therapeutic growth factor in the ischemic heart, as it induces coordinated effects on cardiac blood vessels and cardiomyocytes, ultimately protecting the heart from ischemia.

REVIEW OF THE LITERATURE

Introduction

Ischemic heart disease is the leading cause of death in the modern world, encompassing over seven million deaths per year according to the latest Global Burden of Disease Update of the World Health Organization (World Health Organization, 2008). Angina pectoris symptoms were first systematically described by William Heberden in 1772 (Heberden, 1785), with only isolated cases documented prior to this. This was followed by pathological studies by British scientists who initially described the theory of myocardial ischemia, which however was not fully accepted until over a century later (Proudfit, 1983). During the eighteenth and nineteenth centuries, the condition was rare, and it only became more significant during the early twentieth century, probably due to evolving changes in lifestyle, as first hypothesized by Michaels in 1966 (Michaels, 1966).

Collateral vessels were first described in the human heart in the mid-seventeenth century (Lower, 1669), but considerable controversy about their existence in the normal heart ensued. Post-mortem imaging techniques of animal and human hearts have been developed since the nineteenth century (Spalteholz, 1907; Gross, 1921; Seiler, 2009), and structural coronary anastomoses could be unequivocally visualized in the early 1960's by William Fulton with a novel radiographic technique which permitted visualization of coronary arteries down to fifteen micrometers in diameter (Fulton, 1963a; Fulton, 1963b). Fulton and others were also able to observe not only superficial anastomoses, but also a vast subendocardial arterial plexus arising directly from epicardial arteries (Fulton, 1964) with implications in subendocardial ischemia (Hoffman and Buckberg, 1975), and which could be considerably enlarged in coronary artery disease. Most importantly, the discovery of the existence of structural coronary intercommunications in normal hearts underscored that only their enlargement would be needed for the development of the larger anastomoses found in coronary disease (Fulton and van Royen, 2004).

These observations have led to studies concerning the mechanisms and stimuli behind coronary vessel formation, since although revascularization of stenotic coronary arteries combined with pharmaceutical therapy is still the standard therapy for coronary artery disease, some patient groups respond poorly to these treatments, and the mortality benefit is not clear (Adamu et al., 2010). Therefore, new therapeutic strategies for promoting collateral artery formation, or arteriogenesis, are needed, as well as novel treatments for improving myocardial function. Studies of potential growth factors mediating coronary vessel growth have invariably overlapped with angiogenesis research in for example tumor biology and embryology; thus it is important to review these in the same context.

1. The cardiovascular system

The blood vascular system consists of the heart and a hierarchical network of blood vessels. The heart pumps oxygenated blood first *via* the aorta and major arteries to smaller arterioles and capillaries, where diffusion of oxygen, nutrients and waste products is possible. The deoxygenated blood is then delivered back to the heart *via*

venules and veins and from there to the lungs, where respiration, ventilation and reoxygenation of the blood occur.

1.1. Development of the blood vascular system

This blood circulatory system is the first organ system to develop and appears during the third week of development in the human embryo when passive diffusion of nutrients and waste products becomes insufficient for development (Sadler, 2006). Vasculogenesis, referring to the initial formation of the primitive vascular plexus, begins when hemangioblast progenitors of mesodermal origin migrate and differentiate to form primary blood islands, from which both hematopoietic cells and angiogenic endothelial cell (EC) precursors are formed (Risau and Flamme, 1995). The primitive vascular plexus is subsequently remodeled into a network consisting of arteries, veins, and capillaries of different sizes, and the vessels are stabilized by recruited mural cells, or pericytes and smooth muscle cells (SMCs). Among the most extensively established arterial and venous cell surface markers expressed during early stages of arterial-venous differentiation are the membrane-bound ephrin-B2 ligand and the EphB4 receptor, respectively (Wang et al., 1998).

Angiogenesis, or the process of blood vessel formation from pre-existing vessels, occurs through either sprouting or intussusception (splitting) (see also Risau, 1997; reviewed in Chung and Ferrara, 2011). Vascular endothelial growth factor (VEGF) and its receptors are essential for the early development of the vasculature, whereas later stages of vascular remodeling require for example the angiopoietins and their Tie receptors for maintenance of vascular integrity (reviewed in Saharinen et al., 2010).

Angiogenesis occurs subsequently in the fully developed adult organism during for example wound healing, in the endometrium throughout the menstrual/estrous cycle, in pregnancy, as well as in inflammation and skeletal muscle growth. In addition, angiogenesis plays an important role in several pathological conditions such as cancer and atherosclerosis, as well as in ocular pathologies such as age-related macular degeneration and diabetic retinopathy (Chung and Ferrara, 2011).

1.2. Development of the coronary vasculature

The embryonic origin of coronary ECs is still subject to considerable controversy. One traditional theory involves the proepicardial organ, a transient mesothelial cell structure situated on the surface of the embryonic heart from which epicardial precursors arise. Coronary SMCs and myocardial fibroblasts are thought to be mostly derived from epicardium-derived cells *via* epithelial-to-mesenchymal transition, migrating into the heart along with the epicardium (Mikawa and Gourdie, 1996; Dettman et al., 1998; Vrancken Peeters et al., 1999). A population of coronary ECs has been reported to arise from proepicardial precursors at least in avian embryos (Perez-Pomares et al., 2002). However, more recent studies suggest that the proepicardium is not a major source of ECs (Winter and Gittenberger-de Groot, 2007; Cai et al., 2008).

Interestingly, a novel theory on coronary EC origin has recently been presented in mice, where coronary vessels were shown to sprout from the sinus venosus, the venous endothelial cavity returning blood to the embryonic heart (Red-Horse et al., 2010). In addition, in this study a small population of coronary ECs was shown to originate from

the endocardium, a theory which has also previously been presented although never before supported by clonal analysis (Viragh and Challice, 1981).

1.3. New insights into ischemic heart disease

It has hitherto been generally accepted that ischemic heart disease is pathophysiologically caused by atherosclerotic plaques causing arterial stenosis, although in many patients the severity of symptoms do not correlate with the grade of obstruction. It has recently been suggested that in treating cardiac ischemia, one should not only concentrate on obstructive disease of the coronary arteries themselves, but also on abnormalities of coronary microcirculation, endothelial dysfunction, spontaneous thrombosis and inflammation, all causing dysregulation of blood vessel–cardiomyocyte interactions and capable of leading to myocardial ischemia (Lanza and Crea, 2010; Marzilli et al., 2012). Indeed, novel therapies should perhaps focus on mechanisms which could improve the survival of the cardiomyocytes themselves, regardless of the underlying cause of ischemia.

1.4. Arteriogenesis

Arteriogenesis, a name first proposed by Wolfgang Schaper and colleagues at the end of the twentieth century (Schaper et al., 1999), distinguishes capillary sprouting (angiogenesis) from the growth of collateral vessels able to perfuse an area whose supplying artery has been occluded (Schaper and Schaper, 2004). Fundamental differences exist between the mechanisms behind angiogenesis and those behind collateral vessel growth. For example, hypoxia, a major inducer of angiogenesis, is not required for arteriogenesis, which has been shown to rely instead on mechanical forces such as fluid shear stress, which activates the endothelial and SMC wall of the artery and subsequently recruits mononuclear cells essential for the process (reviewed in Heil and Schaper, 2004; Schaper, 2009). Collateral artery growth is indeed generally understood to result from the remodeling of pre-existing arterial connections, not *de novo* artery formation, since sprouting angiogenesis is usually not seen, and hypoxia-inducible genes do not play a role (Deindl et al., 2001). Indeed, it has been shown in models of hindlimb ischemia that angiogenesis and arteriogenesis occur distinctly, and although tissue ischemia and/or VEGF stimulate capillary sprouting and endothelial cell proliferation, the growth and development of larger collateral vessels with subsequent improved collateral flow occurs when VEGF levels are low (Hershey et al., 2001), suggesting that further signals are needed for SMC proliferation.

It is important to note that many of the same mechanisms are involved during arteriogenesis of smaller arteries as in those leading to atherosclerosis in larger vessels, and many pro-arteriogenic factors have been shown to be also pro-atherogenic (van Royen and Schaper, 2004). Among the features seen in both processes are endothelial activation, increased monocyte chemotactic protein-1 (MCP-1) expression and monocyte recruitment, smooth muscle cell proliferation, and matrix-metalloproteinase (MMP) activation. In addition, angiogenesis and vascular growth factors can contribute to atherosclerotic plaque formation (Celletti et al., 2001; Bhardwaj et al., 2005), putting into question the safety of vascular growth factors for therapeutic angiogenesis. However, the significance of this is not altogether clear, and some studies have indicated that vascular endothelial growth factors have no atherogenic effects (reviewed in Khurana et al., 2005; Leppanen et al., 2005).

Arteriogenesis is also dependent on nitric oxide (NO) signaling, which is also stimulated by fluid shear stress. Of the nitric oxide synthases, inducible nitric oxide synthase (iNOS) seems to be the most important for arteriogenesis (Troidl et al., 2010). Interestingly, endothelial nitric oxide synthase (eNOS) seems not to be required for arteriogenesis after distal femoral artery ligation, but rather for angiogenesis and vasodilation (Murohara et al., 1998; Mees et al., 2007). Among other factors involved in arteriogenesis and arterial remodeling, Notch signaling plays an important role in developmental coronary artery maturation, postnatal arteriogenesis and for maintaining vessel integrity (Liu et al., 2003; van den Akker et al., 2008; Cristofaro et al., 2013), involving also the arterial cell surface marker ephrinB2 (Limbourg et al., 2007; Korff et al., 2008).

As mentioned, the recruitment and activation of mononuclear cells are essential for arteriogenesis (Polverini et al., 1977; Arras et al., 1998). Recently, an important pro-arteriogenic function has been implicated for a special subtype of macrophages, so-called "M2-like" macrophages, as opposed to "M1" macrophages supporting proinflammatory processes (Nucera et al., 2011; Takeda et al., 2011; Hamm et al., 2013).

2. Regulators of myocardial growth, angiogenesis and metabolism

The human heart makes up only about 0.5% of the total body weight but uses 10% of the body's total oxygen consumption and 4% of the total cardiac output (Taegtmeyer, 2007). The heart is a plastic organ, capable of adapting to environmental changes *via* for example myocardial growth or cellular metabolic changes (Hill and Olson, 2008). Cardiac hypertrophy, meaning a thickening of the myocardium, is traditionally divided into physiological *vs.* pathological hypertrophy (reviewed in Dorn, 2007). The latter is usually a result of pressure overload-induced hemodynamic stress and ultimately contributes to heart failure, whereas physiological hypertrophy, most often referring to aerobic exercise-induced cardiac hypertrophy, is by definition a benign and even reversible state. Physiological hypertrophy is seen for example during pregnancy, where the heart adapts to a 40% increase in blood volume and a 45% increase in cardiac output (Hunter and Robson, 1992; Duvekot and Peeters, 1994; Schannwell et al., 2002). However, there is a fine line between physiological and pathological cardiac changes in athletes (Pelliccia et al., 1999; Maron and Pelliccia, 2006), and it has been reported that deconditioning does not always completely reverse exercise-induced cardiac remodeling (Pelliccia et al., 2002). It is also important to note that in athletes, strength training and aerobic conditioning each cause distinct hemodynamic changes (*i.e.* pressure overload *vs.* volume overload) with ultimately different effects on cardiac remodeling (reviewed in Maron and Pelliccia, 2006).

Among the known intracellular signaling pathways involved in cardiac hypertrophy are mitogen-activated protein kinase (MAPK) signaling (Bueno et al., 2000), the phosphoinositide 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) pathway (Shioi et al., 2000; Condorelli et al., 2002; Shioi et al., 2003; Patrucco et al., 2004), calcineurin–NFAT (nuclear factor of activated T-cells) signaling (Molkentin et al., 1998; Wilkins and Molkentin, 2004), as well as other calcium-dependent kinases such as calmodulin-dependent protein kinases (Passier et al., 2000). At the crossroads, glycogen synthase kinase-3β (GSK-3β) plays a modulatory role inhibiting both physiological as well as pathological cardiac growth (Figure 1) (Antos et al., 2002;

reviewed in Kerkela et al., 2007). Interestingly, gene deletion of the cytoplasmic bone marrow kinase in chromosome X (Bmx), a non-receptor tyrosine kinase expressed in arterial endothelium and the endocardium (Ekman et al., 1997; Rajantie et al., 2001) can protect mice from cardiac hypertrophy induced by aortic constriction (Mitchell-Jordan et al., 2008).

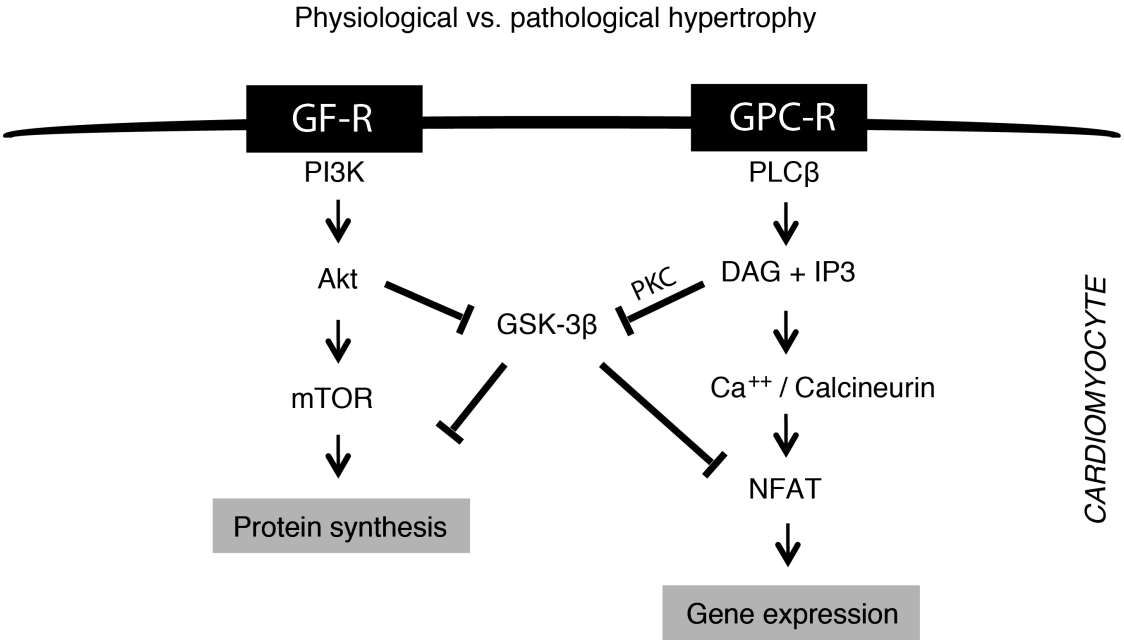

Figure 1. Intracellular signaling pathways associated with physiological (left) vs. pathological (right) cardiac hypertrophy. GF-R, growth factor receptor; PI3K, phosphoinositide 3-kinase; GSK-3β, glycogen synthase kinase-3β; mTOR, mammalian target of rapamycin; GPC-R, G-protein coupled receptor; PLCβ, phospholipase Cβ; PKC, protein kinase C; DAG, diacyglycerol; IP3, inositol triphosphate; NFAT, nuclear factor of activated T-cells. (Adapted from Dorn, 2007; Maillet et al., 2013.)

Prolonged pathological cardiac hypertrophy ultimately leads to decompensation, systolic dysfunction and heart failure (Hill and Olson, 2008). However, synchronized cardiac angiogenesis in animal models of hypertrophy has been shown to be important for preserving cardiac function (Shiojima et al., 2005; Sano et al., 2007). In addition, myocardial hypertrophy can also be induced in mice by angiogenic growth factors alone, where NO signaling seems to play a role (Tirziu et al., 2007; Jaba et al., 2013).

Although the heart relies on mainly fatty acids and glucose as a fuel source, it is capable of burning lactate, ketone bodies and even amino acids (Taegtmeyer, 2007). The heart is very sensitive to changes in blood perfusion, and a reduction in blood flow of only 10-20% can result in exhaustion of the heart's available adenosine triphosphate (ATP) pool. Therefore, flexibility between substrates is crucial in stressed conditions. One important regulator of cardiac metabolism is adenosine monophosphate-activated protein kinase (AMPK), which senses decreases in energy levels and coordinates nutrient uptake and utilization accordingly (reviewed in Zaha and Young, 2012; Maillet et al., 2013).

3. The vascular endothelial growth factor family

The VEGF family consists of five secreted dimeric glycoprotein growth factors in mammals, VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D and PlGF (placenta growth factor). VEGFs belong to the platelet-derived growth factor (PDGF)/VEGF superfamily of growth factors, all containing a VEGF/PDGF homology domain with eight conserved cysteine residues involved in inter- and intramolecular disulfide bond formation. The VEGF ligands are major regulators of blood and lymphatic vessel development and growth and bind with differing specificities to three mainly endothelial transmembrane tyrosine kinase receptors, VEGFR-1/fms-like tyrosine kinase 1 (Flt1), VEGFR-2/human kinase insert domain receptor (KDR)/mouse fetal liver kinase 1 (Flk1) and VEGFR-3/fms-like tyrosine kinase 4 (Flt4). VEGFs also interact with neuropilins (NRP) -1 and -2 (Figure 2A) (reviewed in Pellet-Many et al., 2008).

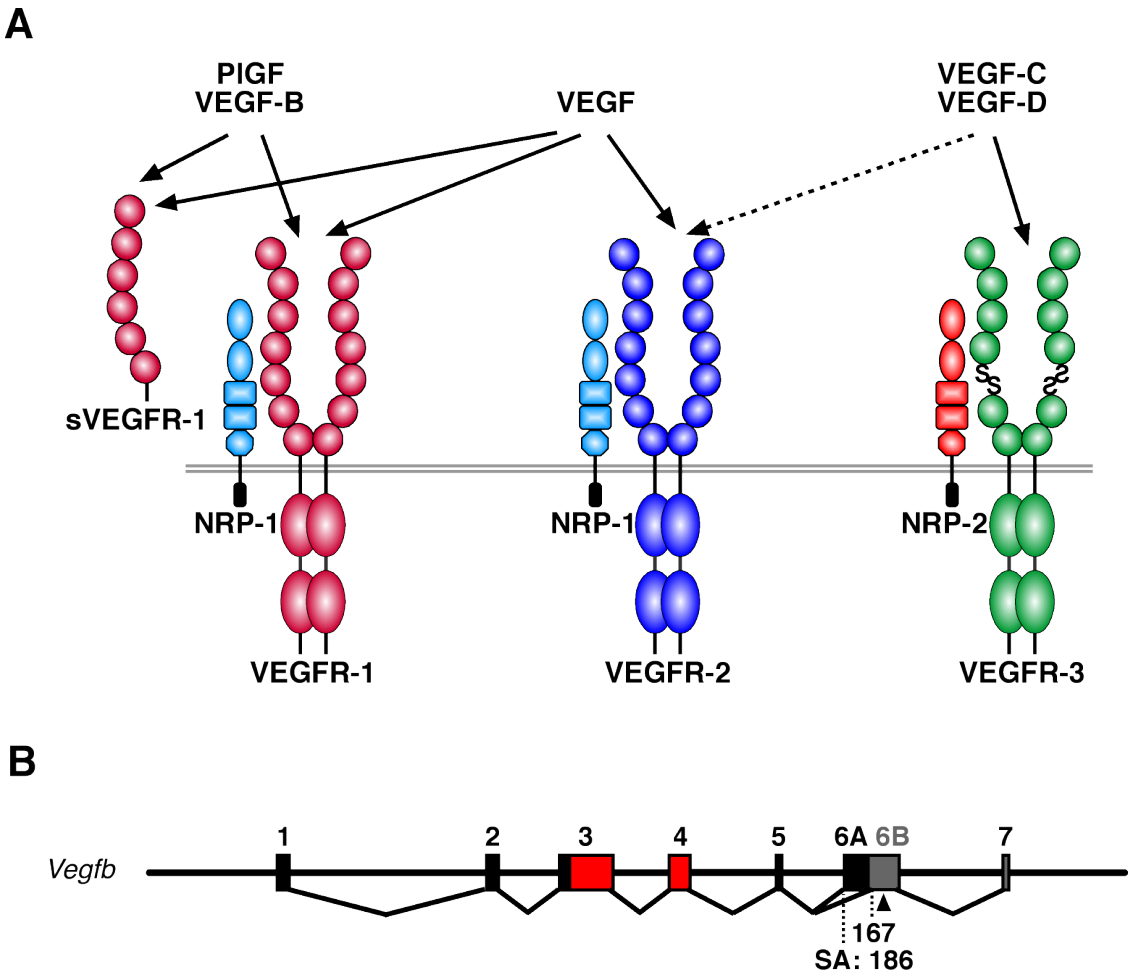

Figure 2. The VEGF family. A. Structure and specific binding of VEGFs to their receptors. The dashed line indicates that processing is required before VEGF-C and human VEGF-D can bind to VEGFR-2. (Adapted from Lohela et al., 2009.) **B.** Schematic structure of the *Vegfb* gene. Shown are exons (numbered) and introns with the alternative splice acceptor (SA) sites that produce the VEGF-B_{167} and VEGF-B_{186} isoforms, where exon 6A is lacking from VEGF-B_{167} mRNA. The arrowhead indicates the site of proteolytic processing of VEGF-B_{186}. Red, sequence encoding the VEGF homology domain.

3.1. VEGF

VEGF, the archetypal angiogenic growth factor, was first identified as a permeability-inducing factor secreted by tumor cells (Senger et al., 1983) and later as a growth factor for vascular endothelial cells (Ferrara and Henzel, 1989; Leung et al., 1989). VEGF is essential for the development of the vasculature, as mice lacking even a single *Vegfa* allele die at embryonic day (E) 11-12 as a result of impaired angiogenesis and blood island formation (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF binds to receptors VEGFR-1 and VEGFR-2 (De Vries et al., 1992; Quinn et al., 1993), as well as to NRP-1 and NRP-2 (Soker et al., 1998; Gluzman-Poltorak et al., 2000). VEGF exists as several splice isoforms, of which $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (in humans) are preferentially expressed (Robinson and Stringer, 2001).

In addition to functioning as a mitogen for ECs, VEGF also regulates EC survival (Alon et al., 1995; Benjamin and Keshet, 1997; Benjamin et al., 1999), mediated through the PI3K–Akt pathway and by inducing the expression of anti-apoptotic proteins (Gerber et al., 1998a; Gerber et al., 1998b). VEGF is also a potent inducer of vascular permeability and inflammation (Gavard and Gutkind, 2006; Nagy et al., 2008). Akt also phosphorylates and activates eNOS, stimulating in turn vasodilation, permeability and angiogenic processes (Fulton et al., 1999; Fukumura et al., 2001; Yu et al., 2005). VEGF also has effects on non-ECs, for example bone marrow derived cells (Clauss et al., 1990; Broxmeyer et al., 1995) and type II pneumocytes (Compernolle et al., 2002).

Overexpression or administration of VEGF results in robust angiogenesis in various tissues (Leung et al., 1989; Isner et al., 1996; Kenyon et al., 1996; Detmar et al., 1998; Larcher et al., 1998; Pettersson et al., 2000), but as mentioned above, it also increases vascular leakage and inflammation (Larcher et al., 1998; Xia et al., 2003), which has hindered its use for therapeutic angiogenesis.

VEGF is upregulated in hypoxia *via* hypoxia inducible factor (HIF)-1α mediated transcription (Schweiki et al., 1992; Forsythe et al., 1996; Pugh and Ratcliffe, 2003). On the other hand, several growth factors, inflammatory cytokines, oncogenes and hormones have also been reported to induce VEGF (reviewed in Ferrara et al., 2003). Interestingly, nutrient and oxygen deprivation also induce the potent metabolic regulator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which is able to induce VEGF independently of HIF-1α in skeletal muscle (Arany et al., 2008), highlighting the close coordination of blood supply and nutrient demand.

3.2. VEGF-B

VEGF-B (previously also known as VRF, VEGF-related factor), first discovered in 1996, has a structure very similar to that of VEGF, and mouse VEGF-B shares approximately 43% amino acid sequence identity with mouse $VEGF_{164}$ (Grimmond et al., 1996; Olofsson et al., 1996a). The gene encoding VEGF-B localizes to chromosome 11q13 in humans, chromosome 19B in mice and chromosome 1q43 in rats (Paavonen et al., 1996; Gerace et al., 2001; Gibbs et al., 2004), and is highly conserved in mammals with about 88% homology at the amino acid level between the mouse and human growth factors (Olofsson et al., 1996a). A primitive form of VEGF-B has also been found in frogs with only 26% homology to human VEGF-B, but

VEGF-B has not been identified in zebrafish (Ruiz de Almodovar et al., 2009). *Vegfb* consists of seven exons and generates two isoforms because of the existence of alternative splice acceptor sites in exon 6 (Figure 2B) (Grimmond et al., 1996; Olofsson et al., 1996b). VEGF-B_{167} has a highly basic heparin-binding carboxy-terminus, whereas VEGF-B_{186} contains a hydrophobic carboxy-terminus and is modified by O-glycosylation and proteolytic processing (Olofsson et al., 1996b). VEGF-B_{167} thus binds tightly to heparan sulfate proteoglycans on the cell surface and in the extracellular matrix, whereas VEGF-B_{186} is freely diffusible. The molecular weights of homodimers of VEGF-B_{167} and VEGF-B_{186} are 42 and 60 kDa, respectively. Both isoforms are simultaneously expressed in various tissues and bind to VEGFR-1 and NRP-1, but not to the major mitogenic endothelial cell receptors VEGFR-2 and VEGFR-3 (Olofsson et al., 1998b; Makinen et al., 1999). Proteolytic processing of VEGF-B_{186} is required for its binding to NRP-1 (Makinen et al., 1999). In culture, VEGF-B is also able to form heterodimers with VEGF (Olofsson et al., 1996a), but this interaction has not been observed *in vivo*.

Unlike VEGF, the expression of VEGF-B does not seem to be directly regulated by hypoxia (Enholm et al., 1997), although hypoxia appeared to induce VEGF-B in the mouse retina in a recent report (Singh et al., 2013). VEGF-B has a wide tissue distribution in mice, being most abundant in tissues with high metabolic activity such as the myocardium, skeletal and vascular smooth muscle, as well as in brown adipose tissue, the brain, kidney, and parietal cells of the stomach (Olofsson et al., 1996a; Lagercrantz et al., 1998; Aase et al., 1999; Capoccia et al., 2009). This could suggest a role for VEGF-B in coordinating the crosstalk between endothelial cell growth and metabolism.

3.2.1. VEGF-B in angiogenesis

Although initial reports indicated that VEGF-B is able to stimulate EC growth *in vitro* (Olofsson et al., 1996a), the ability of VEGF-B to stimulate angiogenesis directly is poor in most tissues. VEGF-B did not stimulate vessel growth when delivered into skeletal muscle or periadventitial tissue with adenoviral vectors (Bhardwaj et al., 2003; Rissanen et al., 2003). VEGF-B also did not improve vascular growth in the ischemic limb (Li et al., 2008a; Lahteenvuo et al., 2009), although some results to the contrary have also been published (Silvestre et al., 2003; Wafai et al., 2009). On the other hand, VEGF-B overexpressed in endothelial cells of transgenic mice was able to potentiate rather than initiate angiogenesis, and unlike VEGF, VEGF-B did not increase vascular permeability (Mould et al., 2005). Overexpression of VEGF-B has similarly been shown to aggravate pathological retinal and choroidal neovascularization in mice (Zhong et al., 2011), and VEGF-B has also been implicated in pathological vascular changes in inflammatory arthritis (Mould et al., 2003). Furthermore, VEGF-B has also been proposed by some to be a survival factor for ECs, regulating the expression of vascular pro-survival genes *via* both NRP-1 and VEGFR-1 (Zhang et al., 2009).

3.2.2. VEGF-B in the heart

Mice deficient of VEGF-B are viable and fertile and display only mild phenotypes in the heart. This is manifested as an atrioventricular conduction abnormality characterized by a prolonged electrocardiographic PQ interval in one strain (Aase et al., 2001), or as a smaller heart size with slightly dysfunctional coronary vasculature

and impaired recovery after myocardial ischemia in another (Bellomo et al., 2000). The latter mouse strain also showed resistance to development of pulmonary hypertension and vascular remodeling during chronic hypoxia (Wanstall et al., 2002). Collectively, the results from models of gene deletion suggest a role for VEGF-B in cardiovascular pathologies.

Interestingly, VEGF-B is expressed in spatial and temporal correlation with the commencement and progression of coronary endothelial growth in the heart, suggesting that it plays a role in coronary vessel development (Bellomo et al., 2000). Importantly, antibodies against VEGF-B were found to inhibit coronary artery development in the quail embryo (Tomanek et al., 2002; Tomanek et al., 2006).

Several studies have indicated a role for VEGF-B in cardiac angiogenesis and/or cardioprotection. VEGF-B levels were found to decrease following experimentally induced myocardial infarction (MI) in rats as well as in heart failure following transverse aortic constriction (Huusko et al., 2012; Zhao et al., 2012). In human patients, low VEGF-B levels were found to accurately predict left ventricular dysfunction and remodeling following MI, suggesting that VEGF-B could be used as a prognostic biomarker with stronger predictive value than troponin T (Devaux et al., 2010; Devaux et al., 2012). Interestingly, the opposite was true for PlGF, as increased PlGF levels predicted heart failure (Devaux et al., 2010).

In experimental models, VEGF-B overexpression has been achieved mainly with adenoviruses or adeno-associated viruses (AAVs). An overdose of VEGF-B_{186} *via* transient adenoviral delivery into the pig myocardium enlarged myocardial vessels after acute infarction, which was inhibited by administration of either soluble VEGFR-1 or soluble NRP-1, but not by blocking VEGFR-2 signaling or NO production (Lahteenvuo et al., 2009). Adenoviral delivery of VEGF-B_{167} enlarged capillaries and ameliorated angiotensin II-induced diastolic dysfunction, with activation of the PI3K–Akt pathway (Serpi et al., 2011). Adenoviral delivery of VEGF-B_{186} was also able to enlarge myocardial capillaries in mice (Huusko et al., 2010). However, AAV-mediated administration of VEGF-B_{167} preserved cardiac contractility and prevented cardiomyocyte apoptosis after experimental myocardial infarction in rats without significant vascular effects (Zentilin et al., 2010). Similar results were achieved in dogs subjected to tachypacing-induced development of dilated cardiomyopathy, where AAV-VEGF-B_{167} administration delayed the progression towards heart failure (Pepe et al., 2010). In addition, a VEGF-B_{186} adenovirus improved systolic function in progressive left ventricular hypertrophy caused by transverse aortic constriction in mice (Huusko et al., 2012). Indeed, the above studies and others (Li et al., 2008a) have implicated the heart as a specific target for VEGF-B induced effects. However, the mechanisms behind these effects are still largely unclear.

3.2.3. Additional roles for VEGF-B

Other studies have implicated a role for VEGF-B in neuroprotection during cerebral ischemia as well as in animal models of neuropathy (Sun et al., 2004; Poesen et al., 2008; Falk et al., 2009), seemingly involving VEGFR-1 expressed in neurons (Li et al., 2008b; Dhondt et al., 2011). VEGF-B expression is also upregulated in some tumor types, such as in ovarian, colorectal, renal cell and prostate cancer (Gunningham et al., 2001; Hanrahan et al., 2003), but its role in tumor progression is poorly

understood. Surprisingly, VEGF-B deficiency in the RIP1-Tag2 mouse model of pancreatic endocrine adenocarcinoma led to increased tumor size, whereas transgenic overexpression of VEGF-B_{167} suppressed tumor growth (Albrecht et al., 2010).

Recent work has investigated a role for VEGF-B in cellular and whole-body metabolism, as VEGF-B is highly expressed in metabolically active tissues. Interestingly, similarly to what was previously published for VEGF (Arany et al., 2008), VEGF-B expression in skeletal muscle is induced by the transcription factor PGC-1α (Bostrom et al., 2012). In addition, it has been shown that VEGF-B expression and a nuclear-encoded mitochondrial gene cluster set are coordinately regulated (Mootha et al., 2003; Hagberg et al., 2010). On the other hand, the absence of VEGF-B was reported to lead to decreased expression of fatty acid transport proteins (FATPs) in endothelial cells, which correlated with decreased amounts of lipid droplets in cardiomyocytes and skeletal muscle fibers, and accumulation of fat in white adipose tissue (Hagberg et al., 2010). Interestingly, blocking VEGF-B improved insulin sensitivity in mouse and rat models of type II diabetes (Hagberg et al., 2012).

3.3. PlGF

PlGF was originally found in the human placenta shortly after the discovery of VEGF (Maglione et al., 1991) and exists as four isoforms in humans but only one in mice (PlGF-2) (Maglione et al., 1993; Cao et al., 1997; Yang et al., 2003). In addition to the placenta, PlGF is expressed in for example the heart, lungs and skeletal muscle (reviewed in De Falco, 2012). PlGF is very similar to VEGF-B in many respects, but the effects of the two growth factors on angiogenesis and arteriogenesis seem to be considerably different. According to most reports, PlGF binds to the same receptors as VEGF-B, namely VEGFR-1 and NRP-1 (Park et al., 1994; Migdal et al., 1998). PlGF gene-targeted mice are viable, but their angiogenesis and arteriogenesis are impaired in ischemia, inflammation and wound healing, as well as in the hypoxic brain (Carmeliet et al., 2001; Freitas-Andrade et al., 2012). Unlike VEGF-B, PlGF is able to stimulate angiogenesis and collateral growth for example in the ischemic heart and limb with similar efficiency to VEGF (Luttun et al., 2002) and also increases vessel permeability (Odorisio et al., 2002) and inflammation (Oura et al., 2003; Selvaraj et al., 2003). However, in a recent report, intracranial PlGF administration with AAV vectors strongly stimulated angiogenesis and arteriogenesis in the brain without significant inflammation or edema (Gaal et al., 2013). PlGF also stimulates the migration and survival of endothelial cells (Ziche et al., 1997; Adini et al., 2002) and can increase the proliferation of smooth muscle cells (Bellik et al., 2005).

It has been reported in numerous studies that PlGF regulates intermolecular crosstalk between VEGFR-1 and VEGFR-2 and enhancement of VEGF signals *via* VEGF/PlGF heterodimer formation (Park et al., 1994; DiSalvo et al., 1995; Cao et al., 1996; Carmeliet et al., 2001; Autiero et al., 2003). However, PlGF is also capable of inducing unique signals through VEGFR-1 (Landgren et al., 1998; Autiero et al., 2003; Schoenfeld et al., 2004). Some studies have suggested that PlGF mediates its arteriogenic effects *via* recruitment of growth factor-secreting monocytes (Pipp et al., 2003).

Much remains to be learned about the mechanisms of PlGF-induced vascularization. The role of PlGF in tumor angiogenesis is also under considerable controversy

(Fischer et al., 2007; Bais et al., 2010; Yao et al., 2011). Interestingly, in the mouse heart, PlGF is able to induce myocardial angiogenesis and cardiac hypertrophy *via* the Akt–mTORC1 pathway, requiring nitric oxide signaling (Jaba et al., 2013). However, in another report, PlGF was only able to support pressure overload-induced cardiac hypertrophy secondarily through a paracrine mechanism *via* endothelial cells and fibroblasts to induce capillary growth and fibroblast proliferation (Accornero et al., 2011).

3.4. VEGFR-1

VEGFR-1 is composed of an extracellular domain, transmembrane domain, intracellular tyrosine kinase (TK) domain, and carboxy-terminal region (Shibuya et al., 1990). The extracellular domain consists of seven immunoglobulin-like (Ig) domains (Figure 2A), with the ability to bind ligands in the second and third Ig domains (Tanaka et al., 1997). VEGFR-1 binds VEGF, VEGF-B and PlGF with high affinity (De Vries et al., 1992). Mice lacking VEGFR-1 produce an excess of ECs and disorganized vasculature and die *in utero* at E8.5-9, suggesting that VEGFR-1 is mainly a negative regulator of angiogenesis, by preventing binding of VEGF to VEGFR-2 (Fong et al., 1995; Fong et al., 1999). This is consistent with the fact that mice engineered to express a truncated form of VEGFR-1, lacking the TK domain responsible for signaling, are viable (Hiratsuka et al., 1998). Similarly, the deletion of VEGFR-1 in adult mice resulted in endothelial cell proliferation and protected against myocardial infarction at least in part *via* upregulation of VEGFR-2 signaling (Ho et al., 2012).

The tyrosine kinase activity of VEGFR-1 in cultured endothelial cells is weak, and its downstream signaling is poorly understood (Waltenberger et al., 1994; Seetharam et al., 1995; Shibuya, 2006). On the other hand, in addition to ECs, VEGFR-1 is expressed at least on monocytes/macrophages and pericytes, and activation of its TK domain is required for monocyte activation and migration (Barleon et al., 1996; Clauss et al., 1996). VEGFR-1 is also expressed in neurons (Poesen et al., 2008). A soluble form of the VEGFR-1 extracellular domain (sVEGFR-1), which is able to neutralize VEGF (Kendall and Thomas, 1993), has been shown to be involved in the pathogenesis of pre-eclampsia (Levine et al., 2004; Kanasaki et al., 2008). Interestingly, VEGFR-1 is expressed also in the endothelium of coronary vessels in the fetal human heart, whereas VEGFR-2 is not, suggesting that VEGFR-1 could play a role in coronary vessel development (Kaipainen et al., 1993; Partanen et al., 1999). In addition, VEGFR-1 expression is upregulated by hypoxia *via* HIF-1α binding regulatory sequences, in contrast to VEGFR-2 and VEGFR-3 (Gerber et al., 1997; Zentilin et al., 2010).

The crystal structure of VEGF-B in complex with VEGFR-1 has been described (Iyer et al., 2006; Iyer et al., 2010), revealing structural interaction with domain 2 of VEGFR-1 similarly to that of VEGF and PlGF. VEGF and PlGF also require domain 3 of VEGFR-1 for high-affinity binding (Davis-Smyth et al., 1998; Christinger et al., 2004). However, it was recently shown that VEGF-B differs in its binding to VEGFR-1 in that it does not require binding to domain 3 (Anisimov et al., 2013a). Receptor specificity of VEGF ligands is determined by an amino-terminal α-helix and three peptide loops, and in this study, it was shown that VEGF-B is unable to induce efficient receptor dimerization and signaling downstream of VEGFR-1 as a result of

the unique structure of loop 1 of VEGF-B. Importantly, swapping loop 1 from PlGF to VEGF-B conferred the angiogenic properties of PlGF to the resulting chimera. These results suggest that VEGF-B–VEGFR-1 signaling is in itself weak, and the effects induced by VEGF-B may at least partly occur through inhibiting other ligands from interacting with VEGFR-1, thus inducing more efficient interaction of VEGF with the highly mitogenic VEGFR-2.

3.5. VEGFR-2

VEGFR-2 is the major receptor mediating VEGF-induced angiogenesis by inducing the proliferation, survival, sprouting and migration of ECs, and also by increasing endothelial permeability (Meyer et al., 1999; Gille et al., 2001). VEGFR-2 is structurally similar to VEGFR-1, but although VEGF binds to VEGFR-2 with a lower affinity than to VEGFR-1, the TK activity of VEGFR-2 is much stronger (Waltenberger et al., 1994; Gille et al., 2000). VEGFR-2 is expressed mainly in ECs, and mice lacking VEGFR-2 die at E8.5-9.5 as a result of impaired vasculogenesis and hematopoiesis (Shalaby et al., 1995).

Downstream signaling by VEGFR-2, following binding of VEGF and receptor dimerization and autophosphorylation of several tyrosine kinase residues, involves numerous pathways, including the phospholipase C (PLC)-γ/protein kinase C (PKC) pathway, causing activation of the c-Raf–MEK–MAP kinase cascade (Guo et al., 1995; Xia et al., 1996; Takahashi et al., 1999), as well as of the PI3K–Akt pathway driving cell survival and migration and eNOS activation (see above under VEGF) (Gerber et al., 1998b).

3.6. VEGF-C, VEGF-D and VEGFR-3

VEGFR-3 is the major receptor controlling lymphangiogenesis, or the growth of lymphatic vessels (reviewed in Tammela and Alitalo, 2010). Different forms of VEGF-C and VEGF-D are produced through proteolytic processing, and their affinity for VEGFR-3 increases with processing. The processed mature forms of human VEGF-C and VEGF-D also bind VEGFR-2 (Joukov et al., 1997; Achen et al., 1998), and heterodimer formation between VEGFR-2 and VEGFR-3 has been reported, particularly in the tip cells of angiogenic vessel sprouts (Dixelius et al., 2003; Olsson et al., 2006; Nilsson et al., 2010). Mouse VEGF-D, on the other hand, binds only to VEGFR-3 (Baldwin et al., 2001). VEGFR-3 is present in all endothelia during early stages of development but becomes restricted to mainly lymphatic endothelial cells in the adult (Kaipainen et al., 1995), although expressed in small amounts also in fenestrated blood capillaries (Partanen et al., 2000). VEGFR-3 is, however, highly expressed in angiogenic sprouts and is involved in postnatal retinal and tumor angiogenesis (Laakkonen et al., 2007; Tammela et al., 2008).

Homozygous deletion of *Vegfc* from mouse embryos leads to a complete absence of lymphatic vessels and embryonic lethality as a result of fluid accumulation in tissues; however, the blood vasculature of these mice appears to develop normally (Karkkainen et al., 2004). VEGF-D deficient mice, on the other hand, are healthy and fertile, and display no obvious defects in lymphatic function (Baldwin et al., 2005). Although loss of VEGFR-3 also results in defective blood vessel development and embryonic death by E9.5 (Dumont et al., 1998), the combined deletion of its ligands VEGF-C and

VEGF-D in mice does not cause any additional defects when compared to the loss of VEGF-C alone (Haiko et al., 2008). This may be explained by roles of VEGFR-3 in ligand-independent signaling and VEGFR-2 heterodimerization (Tammela et al., 2011).

Transgenic overexpression of either VEGF-C or VEGF-D in the skin results in hyperplasia of lymphatic vessels (Jeltsch et al., 1997; Veikkola et al., 2001), but the processed forms of the human growth factors also induce blood vascular growth, likely through VEGFR-2 binding (Saaristo et al., 2002; Rissanen et al., 2003; Anisimov et al., 2009).

3.7. Neuropilins

The neuropilins, NRP-1 and NRP-2, were originally identified as receptors for semaphorins, which mediate repulsive signals during neuronal axon guidance (Chen et al., 1997; Kolodkin et al., 1997). NRP-1 and NRP-2 lack cytoplasmic enzyme activity and function as co-receptors, complexing with other transmembrane receptors such as the VEGFRs (reviewed in Pellet-Many et al., 2008). Neuropilins are also expressed as soluble forms of their extracellular domains and released by cells (Gagnon et al., 2000; Rossignol et al., 2000). VEGF isoforms bind with differing specificity to NRP-1 and NRP-2. $VEGF_{165}$ and $VEGF_{121}$ can reportedly interact with NRP-1 (Soker et al., 1998), whereas $VEGF_{165}$ and $VEGF_{145}$ interact with NRP-2 (Gluzman-Poltorak et al., 2000). PlGF-2 and VEGF-B bind only to NRP-1 (Migdal et al., 1998; Makinen et al., 1999), whereas VEGF-C and VEGF-D can interact with both NRP-1 and NRP-2 (Karkkainen et al., 2001; Karpanen et al., 2006). VEGF can mediate complex formation of VEGFR-2 and NRP-1, which was reported to enhance VEGF/VEGFR-2 interactions (Soker et al., 2002). In addition, both NRP-1 and NRP-2 can form complexes with VEGFR-1 (Fuh et al., 2000; Gluzman-Poltorak et al., 2001), and NRP-2 can interact with VEGFR-3 (Karpanen et al., 2006).

NRP-1 is essential for the formation of the vasculature, as mice lacking NRP-1 die at E13.5 as a result of vascular defects (Kawasaki et al., 1999). NRP-2 gene-targeted mice are viable, but show impaired development of small lymphatic vessels and capillaries in addition to neuronal defects (Yuan et al., 2002). However, embryos lacking both NRP-1 and NRP-2 have an aggravated blood vascular phenotype and die *in utero* at E8.5, implicating compensatory mechanisms between the neuropilins (Takashima et al., 2002). In the heart, NRP-1 is expressed similarly to VEGFR-1 in coronary vessels, myocardial capillaries as well as epicardial vessels (Partanen et al., 1999). Interestingly, NRP-1 is also expressed in the developing myocardium and endocardium in mouse embryos at E12.5 (Makinen et al., 1999).

AIMS OF THE STUDY

This study was undertaken in order to elucidate the role of VEGF-B in the heart as well as its therapeutic potential, focusing on its effects on cardiomyocytes and cardiac blood vessels.

The specific aims were:

I To study and compare the effects of overexpression of VEGF-B in the mouse skin and heart, particularly effects of VEGF-B on blood vasculature, lipid metabolism, and heart function.

II To investigate the mechanisms behind VEGF-B action in the heart and improve our understanding of the effects of VEGF-B on cardiovascular physiology with a novel rat model.

III To study the therapeutic potential of VEGF-B-induced vessel growth in the rat heart and examine the effects of VEGF-B loss-of-function in the rat, as well as the downstream signaling pathways involved.

MATERIALS AND METHODS

The materials and methods used in this study are described in detail in the original publications. A summary of the most relevant materials and methods is provided below.

Table. Materials

Mouse line	Description	Used in	Source/reference
Bmx$^{-/-}$	*Bmx* gene deletion	II	(Rajantie et al., 2001)
C57Bl/6J	Wildtype inbred mice	II, III	Charles River Inc.
FVB/N	Wildtype inbred mice	II	Harlan Inc.
K14-VEGF-B	Overexpresses human VEGF-B in basal epidermal keratinocytes	I	I
Vegfb$^{-/-}$	*Vegfb* gene deletion	III	(Bellomo et al., 2000)
VEGFR-1 TK$^{-/-}$	Lacks the tyrosine kinase domain of VEGFR-1	II	(Hiratsuka et al., 1998)
αMHC-VEGF-B	Overexpresses both isoforms of human VEGF-B in cardiomyocytes	II	II
αMHC-VEGF-B$_{167}$	Overexpresses human VEGF-B$_{167}$ in cardiomyocytes	I	I
αMHC-VEGF-B$_{Ex1-5}$	Overexpresses the first five exons of mouse VEGF-B in cardiomyocytes	II	II

Rat line	Description	Used in	Source/reference
Vegfb$^{-/-}$	*Vegfb* gene deletion	III	III
Wistar (HsdBrl:WH)	Wildtype outbred rats	II, III	Harlan Inc.
αMHC-VEGF-B	Overexpresses both isoforms of human VEGF-B in cardiomyocytes	II, III	II

Recombinant virus	Description	Used in	Source/reference
AAV-HSA	Adeno-associated virus that encodes human serum albumin	II, III	II
AAV-PlGF	Adeno-associated virus that encodes mouse PlGF	II	II
AAV-VEGF	Adeno-associated virus that encodes mouse VEGF$_{120}$	II	II
AAV-VEGF-B$_{167}$	Adeno-associated virus that encodes mouse or human VEGF-B$_{167}$	II, III	II
AAV-VEGF-B$_{186}$	Adeno-associated virus that encodes mouse or human VEGF-B$_{186}$	II, III	II

Antigen	Antibody	Used in	Source/reference
CD45 (rat)	Mouse monoclonal	II, III	BD Biosciences
CD45 (mouse)	Rat monoclonal	III	BD Biosciences
Collagen IV (mouse)	Rabbit	I	Cosmo Bio
Dystrophin (human)	Mouse monoclonal (clone Dy8/6C5)	II, III	Novocastra
ED-1/CD68 (rat)	Mouse monoclonal (MCA341R)	II, III	AbD Serotec
F4/80 (mouse)	Rat monoclonal	II	AbD Serotec
Laminin-1	Rabbit anti-serum	I	Päivi Liesi
Neuropilin-1 (rat)	Goat polyclonal (AF566)	I	R&D Systems
PAI-1 (mouse)	Rabbit	II	Peter Andreasen
PECAM-1 (mouse)	Rat monoclonal (clone MEC13.3)	I, II	BD Biosciences
SMA (human)	Mouse monoclonal-Cy3 (clone 1A4)	II, III	Sigma
VEGF-B (human)	Goat polyclonal (AF751)	I, II	R&D Systems
RECA-1 (rat)	Mouse monoclonal (MCA970)	II, III	AbD Serotec
VEGFR-1 (mouse)	Rat monoclonal (5B12)	I, III	ImClone Systems

Animal models (I, II, III)

All experiments involving mice or rats were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines. Transgenic VEGF-B mouse models were generated by injection of expression cassettes into fertilized mouse oocytes of FVB/N background. To generate the K14-VEGF-B transgenic mice (I), DNA from the human VEGF-B gene corresponding to nucleotides 745-5059 of Genbank accession number AF468110 was cloned into the keratin-14 expression vector (kindly provided by Dr. Elaine Fuchs) (Vassar et al., 1989), and one noninitiating upstream ATG was mutated into a GTG. To generate the heart-specific VEGF-B_{167} transgene (I), the recessed 3'-ends of the EcoRI fragment from a human VEGF-B_{167}/pCRII vector (Olofsson et al., 1996a) were filled in with the Klenow fragment of DNA polymerase I and ligated to the SalI-opened and Klenow filled-in α-myosin heavy chain (αMHC) promoter expression vector (a kind gift from Dr. Jeffrey Robbins).

For generation of mice and rats overexpressing both isoforms of human VEGF-B in cardiomyocytes (II, III), a fragment of the human VEGF-B gene was isolated from the K14-VEGF-B construct (I) and cloned into the αMHC promoter expression vector. To generate αMHC-mVEGF-Bex1-5 mice (II), the mVEGF-Bex1-5 fragment (encoding VEGF-B with the following carboxy-terminal amino acid residues: VKPD) was isolated from the mVEGF-Bex1-5-pSubCMV-WPRE vector with MluI, blunted and cloned into the αMHC promoter expression vector. Transgenic animals were generated by microinjection of fertilized oocytes from FVB/N mice or outbred HsdBrl:WH Wistar rats.

VEGF-B gene-deleted rats of Sprague-Dawley background (III) were generated using a zinc-finger nuclease based technique by Sigma Advanced Genetic Engineering Labs, Sigma-Aldrich Biotechnology (St. Louis, Missouri, USA) (Cui et al., 2011). A 22-base pair segment of exon 1 of the rat VEGF-B gene was replaced with a bacterial *lacZ* gene with a nuclear localization signal (kindly provided by Dr. Thomas Quertermous) (Sheikh et al., 2008) following the endogenous Kozak sequence. Wildtype Sprague-Dawley littermates were used as controls.

Histochemistry and immunohistochemistry (I, II, III)

Tissue samples were either frozen in Optimal Cutting Temperature compound for cryosectioning or embedded in paraffin following fixation and dehydration. Paraffin sections were prepared for stainings *via* deparaffination, rehydration and antigen retrieval. For histochemistry, sections were stained with hematoxylin-eosin, resorcin-fuchsin, Herovici's stain for collagen, or Masson's trichrome. For immunostaining of frozen sections, sections were fixed with cold acetone, washed, and blocked in TNB (PerkinElmer) or 5% donkey serum with 0.2% bovine serum albumin. For whole-mount staining of mouse ears or staining of thick 200 μm heart sections, samples were fixed with 4% paraformaldehyde. The primary antibodies used are detailed in the Table. For immunofluorescence, Alexa Fluor 488, 594 and 647-conjugated secondary antibodies (Molecular Probes) were used for detection, and samples were mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole for nuclear staining (Vector Laboratories). For light microscopy, biotinylated secondary antibodies (Vector Laboratories) were used followed by detection using the Tyramide

Signal Amplification kit (NEN Life Sciences/PerkinElmer), 3-amino-9-ethylcarbazole substrate (Sigma-Aldrich), and hematoxylin counterstaining.

Fluorescently labeled samples were imaged using an Axioplan2 fluorescence microscope (Zeiss) or a confocal LSM 510 Meta or LSM 5 Duo microscope (Zeiss). Peroxidase-stained samples were imaged with a Leica DM LB research microscope. Image analyses were carried out using the ImageJ software (National Institutes of Health) from several randomly chosen photographic fields from each sample.

Western blotting (I, II, III)

Tissue samples were sliced into small pieces, mixed with RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 50 mM Tris pH 8.0, 20 μg/mL leupeptin, 3.4 μg/mL aprotinin, 1 mM Na_3VO_4, 1 mM PMSF) in Lysing Matrix tubes (MP Biomedicals), and homogenized. Total protein concentrations were measured using the BCA Protein Assay Kit (Thermo Scientific). Lysates were boiled in Laemmli sample buffer (LSB), and equal amounts of total protein samples were subsequently separated in SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. Antibody complexes were visualized on X-ray film using chemiluminescent substrate (Thermo Scientific).

Transmission electron microscopy (I, II, III)

Tissue samples were fixed in 2.5% glutaraldehyde, postosmicated and embedded in epon. Semithin sections were stained with toluidine blue, and on the basis of initial analysis in light microscopy, regions of interest were selected for thin (100 nm) sectioning and analysis using a JEOL 1400 EX or Philips EM-400 Transmission Electron Microscope.

Blood pressure analysis (I, II)

Heart rate and mean arterial pressure were recorded from the left carotid artery in mice using telemetric implants (Butz and Davisson, 2001) (I). Blood pressure was measured in rats with the CODA Non-Invasive Blood Pressure System for Mice and Rats (Kent Scientific Corporation) on non-anesthetized animals restrained in a rodent holder (II).

Echocardiography (I, II, II)

Transthoracic echocardiography was performed on anesthetized animals with an Acuson Sequoia 512 Ultrasound System and an Acuson Linear 15L8 transducer (Siemens). Normal body temperature was maintained.

Measurements in isolated, perfused hearts (I)

The mouse aorta was cannulated after cervical dislocation and perfusion commenced immediately. The heart was then excised and perfusion continued *ex vivo*. Left ventricular pressure was monitored using a saline-filled cannula connected to a Statham P231D pressure transducer and SP1400 pressure monitor. Venous effluent

from the heart was collected in one-minute aliquots, and the lactate dehydrogenase washout was measured.

Recombinant AAV vector preparation (II, III)

cDNAs were cloned into blunted MluI and NheI restriction sites of the psubCMV-WPRE recombinant AAV expression vector. The recombinant AAVs (serotype 9) were produced as described previously (Anisimov et al., 2009).

Micro-CT imaging of the cardiac vessels (II, III)

Post mortem coronary angiographies were first performed (II) using the Inveon micro-computed tomography (CT) scanner (Siemens). The ascending aorta was cannulated, clamped and filled with 0.3 mL of iodinated intravascular contrast agent eXIATM160XL (Binitio Biomedical Inc.). The coronary arterial and venous trees were segmented using the ADW 4.4 Workstation (General Electric) and visualized as three-dimensional volume rendered images.

High-resolution micro-CT imaging (III) was performed according to Tirziu *et al.* (Tirziu et al., 2005). The aorta was cannulated retrogradely proximal to the brachiocephalic trunk, and the hearts were perfused with heparin (100 IU/kg) followed by adenosine (1 mg/ml) and then perfusion-fixed with 4% paraformaldehyde. The coronary arterial tree was filled with contrast agent consisting of 20% bismuth oxychloride (Sigma-Aldrich) in 5% gelatin, filling only the arterial vessels. Filled hearts were imaged with a high-resolution micro-CT imaging system (GE eXplore Locus SP) followed by morphometric analysis of the arterial vessels.

Experimental myocardial infarction (III)

Myocardial infarction was induced in rats by ligation of the left coronary artery (LCA). Infarct sizes were estimated from Masson's trichrome stained transverse heart sections (Pfeffer et al., 1979).

Positron emission tomography (PET) (II, III)

Rats were given a slow bolus of ^{11}C-acetate (0.4–1.0 ml) and imaged using the Inveon microPET scanner (Siemens). Myocardial blood flow, oxygen consumption and efficiency were estimated from the images. For validation of infarct size measurements (III), a subgroup of the rats was injected in a separate imaging session with ^{18}F-fluorodeoxyglucose, a marker of myocardial glucose metabolism and viability.

Quantitative reverse transcription PCR (III)

Total RNA was isolated from the left ventricle with the TRIsure reagent (Bioline) and further purified with NucleoSpin RNA II (Macherey-Nagel). RNA was transcribed to cDNA using the iScript kit (Bio-Rad), and quantitative PCR was carried out following standard procedures with the SYBR green or TaqMan primer-probe sets. All data were normalized to 18S, β-actin and TBP (TATA-binding protein) housekeeping genes, and quantification was performed using the $2^{-\Delta\Delta CT}$ method.

Microarray analysis (III)

RNA samples were analyzed with the genome-wide Illumina RatRef-12 Expression BeadChip (BD-27-303; Illumina Inc.) or Affymetrix Rat Gene 2.0 chips. Illumina's GenomeStudio software was used for initial data analysis and quality control. Detailed data analyses were performed with the Chipster software (http://www.chipster.csc.fi). After quantile normalization, statistically significant differences in individual genes between the groups were tested using Empirical Bayes statistics and the Benjamin-Hochberg algorithm controlling false discovery rate (FDR). Adjusted FDR values of $P<0.05$ were considered significant.

Lipidomics (I) and metabolomics (III)

For lipidomics (I), lipid extracts were analyzed on a Waters Q-Tof Premier Mass Spectrometer (Waters, Inc.) combined with an Acquity UltraPerformance Liquid Chromatograph. For metabolomics (III), samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method (Metabolon Inc.). The extracted samples were split into equal parts for analysis on the gas chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry platforms. Detection and quantification of malonyl-coenzyme A esters (III) was accomplished by extracting coenzyme A esters from powdered tissue and measuring with a modified high-performance liquid chromatograph.

Statistical analysis (I, II, III)

Values were presented as means ± SD or SEM. Statistical analysis was performed with one-way ANOVA (with Tukey's post-hoc test), the two-tailed unpaired Student's t-test or the Mann-Whitney *U* test, where appropriate. Differences were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

The main results of the study are summarized and discussed here. A detailed discussion can be found in the original publications.

1. A novel role for VEGF-B in cardiac hypertrophy and lipid metabolism (I)

Although VEGF-B is closely related to other members of the VEGF family, its effects on blood vessel growth have been shown to be extremely modest. However, VEGF-B is highly expressed in tissues with active energy metabolism, such as the myocardium, skeletal and vascular smooth muscle, as well as in brown adipose tissue (Olofsson et al., 1996a; Aase et al., 1999). We endeavored to elucidate the possible role of VEGF-B in the heart using transgenic mice expressing heparin-binding human VEGF-B_{167}, which has been published to be the most abundant isoform (Li et al., 2001), under the myocardium-specific α-myosin heavy chain (αMHC) promoter. At the same time, we compared the effect of overexpression of VEGF-B in the skin, using the keratin-14 promoter expressed in basal keratinocytes in the epidermis (Vassar et al., 1989).

Similarly to previously published results for other tissues (*e.g.* Rissanen et al., 2003), VEGF-B did not cause robust angiogenesis in the heart or skin in mice. However, the hearts overexpressing VEGF-B_{167} were larger and heavier than their littermate controls, as calculated from heart-to-body weight ratios. The hypertrophy was associated with an increase in the cross-sectional diameter of the cardiomyocytes, and in transthoracic echocardiography, the VEGF-B overexpressing hearts showed concentric cardiac hypertrophy, with thicker wall dimensions but no change in diastolic left ventricular diameter. Surprisingly, the VEGF-B transgenic mice had lower blood pressure and heart rate, indicating that the cardiac hypertrophy did not result from pressure overload, but that it was due to intrinsic changes in the myocardium.

Although VEGF-B overexpression actually led to a decrease in capillary density as a result of the increased size of cardiomyocytes, the capillaries in VEGF-B transgenic hearts were larger, with an increase in the number of endothelial cells per vessel cross-section. This indicated a mild proliferation of endothelial cells, which however did not lead to an increase in capillary sprouts. Thus the effect of VEGF-B on blood vessels differed from *e.g.* the effects of VEGF.

Echocardiography revealed that the hypertrophy induced by VEGF-B did not cause significant changes in the systolic function of the heart even at twelve months of age. In addition, the VEGF-B transgenic hearts responded similarly to treatment with angiotensin II, indicating that their tolerance of pressure overload was not compromised despite the hypertrophy. Also, no obvious differences in mitochondrial energy coupling or the basal metabolic rate could be seen, and isolated transgenic hearts showed less damage following ischemia-reperfusion, as seen by an initial decrease in the washout of lactate dehydrogenase.

On the other hand, we observed decreased survival of the VEGF-B transgenic mice by six months of age, likely as a result of a propensity toward arrhythmias. Vacuoles could be seen inside cardiomyocytes of the VEGF-B overexpressing hearts in light microscopy at one year of age, seemingly resulting from damaged mitochondria seen

in electron microscopy at earlier time points, although mitochondrial function appeared normal. Associated with these pathological changes was an increase in *de novo* ceramide synthesis, likely a reason for the lysis/autophagy of mitochondria. Interestingly, we also observed a decrease in the amount of triacylglycerols in the VEGF-B transgenic hearts. VEGF-B thus seemed to have a novel function in lipid metabolism, but whether these metabolic effects were a cause or consequence of the hypertrophy induced by VEGF-B could not yet be concluded from this study.

2. VEGF-B as a novel growth factor for coronary arteries (II)

As the signals mediating the effects of VEGF-B in the heart were still unknown, we proceeded with additional animal models to analyze the mechanisms behind the hypertrophy induced by VEGF-B and simultaneously searched for other actions of VEGF-B. We proceeded first to compare the effects of VEGF-B and other members of the VEGF family on blood vessels in mouse skeletal muscle using local injection of recombinant AAV vectors. VEGF-B did not induce blood vessel growth in skeletal muscle during four weeks of expression, but importantly, it also did not increase inflammation or vessel permeability like VEGF or PlGF. Thus, if VEGF-B could be used therapeutically for example in the heart, as has also been suggested by other groups (Li et al., 2008a; Lahteenvuo et al., 2009; Zentilin et al., 2010), toxicity as a result of vascular leakage should not be a major problem.

As rats are better suited for studies of cardiovascular physiology, we proceeded to create a transgenic rat model, this time using the full-length human VEGF-B gene, producing both VEGF-B_{167} and VEGF-B_{186} under the αMHC-promoter. Similarly to what we previously observed for mice, VEGF-B was able to induce cardiac hypertrophy associated with decreased blood pressure and heart rate, as well as capillary enlargement. In contrast to the mouse model however, we could not observe degenerative changes in the rat cardiomyocytes overexpressing VEGF-B. This seemed to be a result of a robust expansion of the coronary arterial tree, including branches extending into the subendocardial region (Figure 3). Coordinated blood vessel growth has indeed been shown to be important for maintaining heart function in cardiac hypertrophy (Shiojima et al., 2005; Sano et al., 2007). Importantly, the subendocardium is the first area to suffer from ischemia as a result of hypertension, coronary artery disease, or aging (Lumens et al., 2006), and the subendocardial coronary arterial plexus has previously been shown to be an important reserve in coronary artery disease (Hoffman and Buckberg, 1975). Although others have shown that strong overexpression of VEGF-B can increase arterial growth following myocardial ischemia (Lahteenvuo et al., 2009), our study was the first to demonstrate that VEGF-B can induce arteriogenesis without any initial ischemic insult. The coronary arteriogenesis was associated with increased expression of plasminogen activator inhibitor (PAI)-1 in arterial smooth muscle. PAI-1 plays an important role in the regulation of matrix degradation during angiogenesis (Pepper, 2001) as well as in myoendothelial junction formation (Heberlein et al., 2010), and VEGF-B has also previously been shown to activate PAI-1 in endothelial cells in culture, similarly to VEGF (Pepper et al., 1991; Olofsson et al., 1998a).

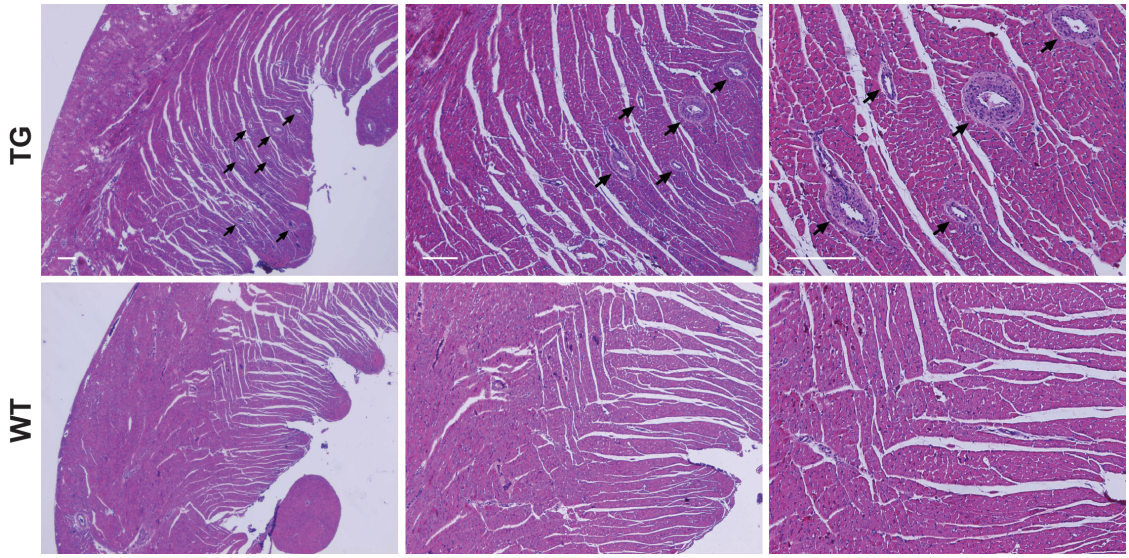

Figure 3. Subendocardial arteriogenesis in VEGF-B transgenic (TG) rat hearts. Representative images of transverse left ventricular sections from transgenic (TG) and wildtype (WT) rats stained with hematoxylin-eosin. Arrows indicate large subendocardial arteries. Scale bars: 200 μm.

Since VEGF-B binds to two receptors, VEGFR-1 and NRP-1 (Olofsson et al., 1998b; Makinen et al., 1999), we next endeavored to elucidate which of these is required for the effects of VEGF-B in the heart using genetic mouse models. We overexpressed a truncated form of VEGF-B containing only the first five exons, with the ability to bind to VEGFR-1 but not to NRP-1 (Makinen et al., 1999). This form of VEGF-B was still able to induce cardiac hypertrophy, indicating that NRP-1 is not required for this effect of VEGF-B. However, the VEGF-B transgene was not able to increase heart size in mice lacking the tyrosine kinase domain of VEGFR-1 (Hiratsuka et al., 1998), indicating that VEGFR-1 signaling is important for the VEGF-B induced cardiac hypertrophy. We proceeded to investigate a possible role for Bmx, a non-receptor tyrosine kinase expressed mainly in the endothelium of large arteries, since VEGFR-1 was previously shown to increase Bmx phosphorylation and TK activity *in vitro* (Rajantie et al., 2001). In addition, Bmx has been implicated by others to be required for pressure overload induced cardiac hypertrophy (Mitchell-Jordan et al., 2008). Crossing the VEGF-B transgenic mice with Bmx deficient mice indeed significantly attenuated the hypertrophy induced by VEGF-B, suggesting that signaling *via* Bmx and the arterial endothelium is involved in the induction of hypertrophy by VEGF-B. Since Bmx and VEGFR-1 are expressed primarily in endothelial cells in the heart (Partanen et al., 1999), additional unknown signals from endothelial cells would be required for effects of VEGF-B on cardiomyocytes.

3. VEGF-B protects the heart from myocardial ischemia and alters cardiac energy metabolism (III)

As the vast coronary arteriogenesis in VEGF-B transgenic rat hearts showed considerable therapeutic promise for this growth factor in the heart, we next endeavored to characterize the phenotype of the rats more closely and test their performance in stressed conditions. We also strove to elucidate the metabolic role of

VEGF-B in the heart, since results from other groups have implicated a role for VEGF-B in endothelial fatty acid uptake (Hagberg et al., 2010; Hagberg et al., 2012), and since our results from mice also indicated some role for VEGF-B in cardiac energy metabolism (I).

High-resolution micro-CT imaging of the heart revealed an expansion of coronary arteries of all sizes in VEGF-B transgenic rats, but mainly in the largest arteries. Importantly for possible therapeutic applications, we were able to reproduce most of this phenotype using recombinant AAV-VEGF-B administered systemically to adult rats. Interestingly, most previous studies describing effects of VEGF-B on blood vessels in the heart have used very robust but transient overexpression *via* adenoviral delivery (Lahteenvuo et al., 2009), whereas we can achieve clear effects with expression levels closer to physiological levels of VEGF-B (about 6-8 fold over endogenous levels).

To test whether the expanded collateral arteries were functional in VEGF-B transgenic hearts, we subjected the rats to myocardial infarction induced by ligation of the left coronary artery. The resulting infarcts were indeed significantly smaller in the VEGF-B transgenic hearts, which showed improved blood perfusion and cardiac function when compared to wildtype hearts. Importantly, the VEGF-B transgenic hearts maintained their systolic function and during aging actually showed increased stroke volume when compared to wildtype hearts, confirming the physiological nature of the cardiac hypertrophy in VEGF-B transgenic rats.

Since we had shown that VEGFR-1 signaling is involved in the induction of hypertrophy by VEGF-B (II), we next undertook to find the mechanisms behind the growth of cardiac vessels. Increased phosphorylation of VEGFR-2 and its downstream targets were observed following intravenous administration of VEGF protein to VEGF-B overexpressing animals, indicating that VEGF-B, while occupying VEGFR-1, is able to increase VEGF–VEGFR-2 signaling. This effect would however not be as robust as the angiogenesis caused by VEGF overexpression, since it would regulate signaling at endogenous, phsyiological VEGF levels. Blocking VEGFR-2 signaling did indeed reduce the vessel enlargement seen in VEGF-B overexpressing mice. Similarly, in VEGFR-1 deficient mice, upregulation of VEGFR-2 has been shown to contribute to increased angiogenesis and protection against ischemic damage (Ho et al., 2012).

We next generated a rat model of VEGF-B gene deletion, and similarly to what has previously been published for mice (Bellomo et al., 2000; Aase et al., 2001), loss of VEGF-B did not have obvious effects on blood vessel development. When the VEGF-B deficient rats were subjected to myocardial infarction, no significant differences in cardiac function could be observed; however, the histologically quantified infarct areas were larger in VEGF-B deficient hearts. Some compensatory mechanisms are thus present in the context of constitutive VEGF-B gene deletion, but loss of VEGF-B does seem to mildly impair the coronary vasculature in stressed conditions, as has been published for isolated VEGF-B knockout mouse hearts subjected to ischemia (Bellomo et al., 2000).

Importantly, we were not able to observe any differences in fatty acid uptake or triglyceride content between the VEGF-B wildtype, transgenic or gene-deleted rat

hearts, challenging an existing theory that VEGF-B regulates endothelial fatty acid uptake (Hagberg et al., 2010; Hagberg et al., 2012). Thus it is clear that the metabolic effects proposed for VEGF-B are not as simple as has been suggested.

In our rat models of VEGF-B overexpression, we could however observe a shift from fatty acid to glucose usage in the heart, while pathways directing lipid/macromolecular synthesis, supportive of cell and vessel growth, were induced. Although a shift towards glycolysis occurs in pathological hypertrophy, in our models this was not accompanied by an increase in genes associated with pathological hypertrophy, and blood supply and nutrient usage remained efficient in our models. The failing heart is indeed characterized by an inefficiency to use either substrate group efficiently, and efficient glucose usage has been suggested to slow the progression of heart failure (Taegtmeyer, 2002). In fact, activation of fatty acid metabolism actually results in contractile failure in the hypertrophied rat heart (Young et al., 2001), and drugs favoring glucose oxidation can protect the ischemic heart (Kantor et al., 2000; Abozguia et al., 2010). In summary, close regulation of metabolic pathways seems to be essential for maintenance of heart function. The various effects of VEGF-B in the rat heart are summarized below (Figure 4).

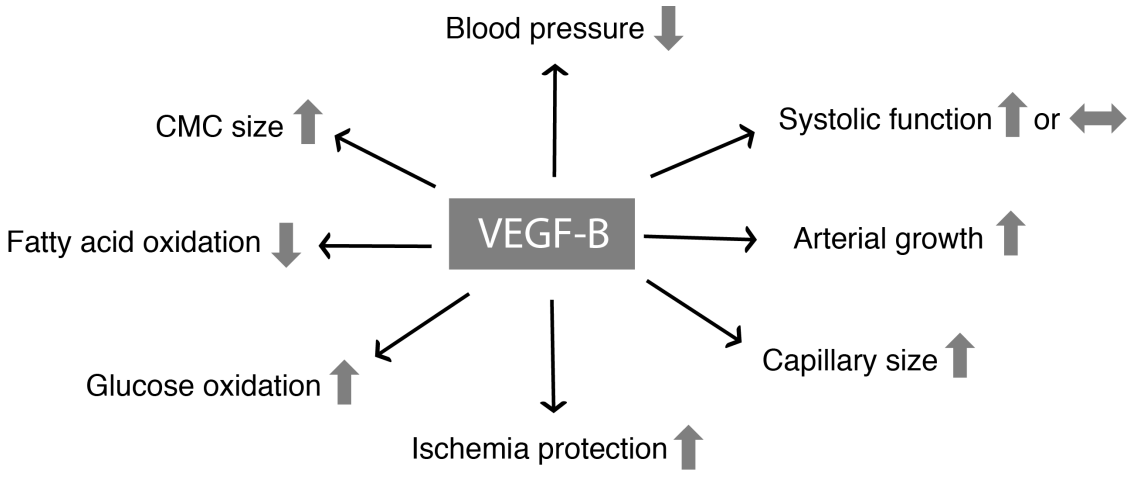

Figure 4. Summary of the effects of VEGF-B in the rat heart. Gray arrows indicate an increase, decrease, or no change. CMC, cardiomyocyte.

CONCLUDING REMARKS

New therapeutic strategies are needed for the treatment of ischemic heart disease, and research on growth factors regulating vascular growth in the heart has recently been fruitful. However, most angiogenic growth factors also increase inflammation and vessel permeability (Lee et al., 2000); thus there are obstacles on the road towards efficient and safe gene therapy for ischemic diseases.

Among VEGFs, PlGF has shown promise in animal models of myocardial ischemia, enhancing regional blood flow and preserving contractile function (Kolakowski et al., 2006; Liu et al., 2013), at least in part through upregulation of VEGF (Lahteenvuo et al., 2009). It is of interest that PlGF seems mainly to increase the growth of capillaries and smaller arterioles in the myocardium (Jaba et al., 2013), whereas in our models, the effects of VEGF-B were strongest in larger arterioles and arteries (III). In addition, adenoviral administration of VEGF-C has been shown to increase collateral vessel formation in a model of myocardial ischemia in pigs (Patila et al., 2006). Adenoviral delivery of VEGF or the mature form of VEGF-D was also able to increase angiogenesis at least in the non-ischemic myocardium; however, increased vessel permeability became an issue at higher doses (Rutanen et al., 2004; Lahteenvuo et al., 2009). So far, in phase II/III clinical trials, intracoronary or intramyocardial VEGF administration has only resulted in limited or no benefit in patients with severe coronary artery disease (reviewed in Yla-Herttuala et al., 2007; Yla-Herttuala, 2013). However, additional trials are ongoing, and it is possible that patients with earlier stage disease would respond better to angiogenic gene therapy, and delivery methods for achieving sufficient growth factor concentrations in the human heart also need to be improved. There is also the possibility of using chimeric growth factors in order to achieve optimal efficiency with fewer side effects (Anisimov et al., 2013b).

Both our results as well as results from other groups have presented VEGF-B as a promising therapeutic vector in experimental models of myocardial ischemia and/or heart failure. In our hands, VEGF-B induces cardiac hypertrophy as well as enlarges coronary arteries and myocardial capillaries in the rat heart. Importantly, VEGF-B does not induce inflammation or vascular leakage. Instead, the effects of VEGF-B on cardiac blood vessels seem to be caused by fine regulation of VEGF–VEGFR-2 signaling by VEGF-B, while the cardiac hypertrophy seems to involve VEGFR-1–Bmx signaling.

Importantly, the hypertrophied VEGF-B transgenic rat hearts maintained improved systolic function also during aging, and they were protected from ischemic damage caused by coronary artery ligation. In mouse hearts, pathological changes could be seen inside cardiomyocytes while the effects on blood vessel growth were milder, suggesting differences in collateral artery formation between the species.

VEGF-B signaling, however, does remain enigmatic. Since its receptors are mainly expressed on blood vascular endothelial cells, additional signals from endothelial cells would be needed for effects on cell growth and metabolism of the cardiomyocytes themselves. It is also conceivable that VEGFR-1 in inflammatory cells could play a role, or additional unknown receptors.

Finally, administration of VEGF-B with AAV-VEGF-B seemed to be efficient and safe. However, the effects of VEGF-B overexpression required two to three weeks of expression before becoming apparent, so it is perhaps not conceivable that future therapies based on VEGF-B would be helpful in acute myocardial infarction, but rather could be beneficial during long-term recovery and in strengthening the myocardium by inducing favorable metabolic changes in cases of ischemic heart disease. The close interplay between angiogenesis and oxidative metabolism will likely be the focus of numerous studies in the near future, and VEGF-B is one potential candidate molecule in this crosstalk.

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REFERENCES

Aase, K., Lymboussaki, A., Kaipainen, A., Olofsson, B., Alitalo, K. and Eriksson, U., 1999. Localization of VEGF-B in the mouse embryo suggests a paracrine role of the growth factor in the developing vasculature. Dev Dyn 215, 12-25.

Aase, K., von Euler, G., Li, X., Ponten, A., Thoren, P., Cao, R., Cao, Y., Olofsson, B., Gebre-Medhin, S., Pekny, M., Alitalo, K., Betsholtz, C. and Eriksson, U., 2001. Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. Circulation 104, 358-364.

Abozguia, K., Elliott, P., McKenna, W., Phan, T.T., Nallur-Shivu, G., Ahmed, I., Maher, A.R., Kaur, K., Taylor, J., Henning, A., Ashrafian, H., Watkins, H. and Frenneaux, M., 2010. Metabolic modulator perhexiline corrects energy deficiency and improves exercise capacity in symptomatic hypertrophic cardiomyopathy. Circulation 122, 1562-9.

Accornero, F., van Berlo, J.H., Benard, M.J., Lorenz, J.N., Carmeliet, P. and Molkentin, J.D., 2011. Placental growth factor regulates cardiac adaptation and hypertrophy through a paracrine mechanism. Circ Res 109, 272-80.

Achen, M.G., Jeltsch, M., Kukk, E., Makinen, T., Vitali, A., Wilks, A.F., Alitalo, K. and Stacker, S.A., 1998. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci U S A 95, 548-53.

Adamu, U., Knollmann, D., Alrawashdeh, W., Almutairi, B., Deserno, V., Kleinhans, E., Schafer, W. and Hoffmann, R., 2010. Results of interventional treatment of stress positive coronary artery disease. Am J Cardiol 105, 1535-9.

Adini, A., Kornaga, T., Firoozbakht, F. and Benjamin, L.E., 2002. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. Cancer Res 62, 2749-52.

Albrecht, I., Kopfstein, L., Strittmatter, K., Schomber, T., Falkevall, A., Hagberg, C.E., Lorentz, P., Jeltsch, M., Alitalo, K., Eriksson, U., Christofori, G. and Pietras, K., 2010. Suppressive effects of vascular endothelial growth factor-B on tumor growth in a mouse model of pancreatic neuroendocrine tumorigenesis. PLoS One 5, e14109.

Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J. and Keshet, E., 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1, 1024-1028.

Anisimov, A., Alitalo, A., Korpisalo, P., Soronen, J., Kaijalainen, S., Leppanen, V.M., Jeltsch, M., Yla-Herttuala, S. and Alitalo, K., 2009. Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle. Circ Res 104, 1302-12.

Anisimov, A., Leppanen, V.M., Tvorogov, D., Zarkada, G., Jeltsch, M., Holopainen, T., Kaijalainen, S. and Alitalo, K., 2013a. The basis for the distinct biological activities of vascular endothelial growth factor receptor-1 ligands. Sci Signal 6, ra52.

Anisimov, A., Tvorogov, D., Alitalo, A., Leppanen, V.M., An, Y., Han, E.C., Orsenigo, F., Gaal, E.I., Holopainen, T., Koh, Y.J., Tammela, T., Korpisalo, P., Keskitalo, S., Jeltsch, M., Yla-Herttuala, S., Dejana, E., Koh, G.Y., Choi, C., Saharinen, P. and Alitalo, K., 2013b. Vascular endothelial growth factor-angiopoietin chimera with improved properties for therapeutic angiogenesis. Circulation 127, 424-34.

Antos, C.L., McKinsey, T.A., Frey, N., Kutschke, W., McAnally, J., Shelton, J.M., Richardson, J.A., Hill, J.A. and Olson, E.N., 2002. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A 99, 907-12.

Arany, Z., Foo, S.Y., Ma, Y., Ruas, J.L., Bommi-Reddy, A., Girnun, G., Cooper, M., Laznik, D., Chinsomboon, J., Rangwala, S.M., Baek, K.H., Rosenzweig, A. and Spiegelman, B.M., 2008. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature 451, 1008-12.

Arras, M., Ito, W.D., Scholz, D., Winkler, B., Schaper, J. and Schaper, W., 1998. Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. J Clin Invest 101, 40-50.

Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F., Kliche, S., Fellbrich, G., Ballmer-Hofer, K., Maglione, D., Mayr-Beyrle, U., Dewerchin, M., Dombrowski, S., Stanimirovic, D., Van Hummelen, P., Dehio, C., Hicklin, D.J., Persico, G., Herbert, J.M., Communi, D., Shibuya, M., Collen, D., Conway, E.M. and Carmeliet, P., 2003. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 9, 936-43.

Bais, C., Wu, X., Yao, J., Yang, S., Crawford, Y., McCutcheon, K., Tan, C., Kolumam, G., Vernes, J.M., Eastham-Anderson, J., Haughney, P., Kowanetz, M., Hagenbeek, T., Kasman, I., Reslan, H.B., Ross, J., Van Bruggen, N., Carano, R.A., Meng, Y.J., Hongo, J.A., Stephan, J.P., Shibuya, M. and Ferrara, N., 2010. PlGF blockade does not inhibit angiogenesis during primary tumor growth. Cell 141, 166-77.

Baldwin, M.E., Halford, M.M., Roufail, S., Williams, R.A., Hibbs, M.L., Grail, D., Kubo, H., Stacker, S.A. and Achen, M.G., 2005. Vascular endothelial growth factor D is dispensable for development of the lymphatic system. Mol Cell Biol 25, 2441-9.

Baldwin, M.E., Roufail, S., Halford, M.M., Alitalo, K., Stacker, S.A. and Achen, M.G., 2001. Multiple forms of mouse vascular endothelial growth factor-D are generated by RNA splicing and proteolysis. J Biol Chem 276, 44307-14.

Barleon, B., Sozzani, S., Zhou, D., Weich, H.A., Mantovani, A. and Marme, D., 1996. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood 87, 3336-43.

Bellik, L., Vinci, M.C., Filippi, S., Ledda, F. and Parenti, A., 2005. Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. Br J Pharmacol 146, 568-75.

Bellomo, D., Headrick, J.P., Silins, G.U., Paterson, C.A., Thomas, P.S., Gartside, M., Mould, A., Cahill, M.M., Tonks, I.D., Grimmond, S.M., Townson, S., Wells, C., Little, M., Cummings, M.C., Hayward, N.K. and Kay, G.F., 2000. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circ Res 86, E29-E35.

Benjamin, L.E., Golijanin, D., Itin, A., Pode, D. and Keshet, E., 1999. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 103, 159-65.

Benjamin, L.E. and Keshet, E., 1997. Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc. Natl. Acad. Sci. USA 94, 8761-8766.

Bhardwaj, S., Roy, H., Gruchala, M., Viita, H., Kholova, I., Kokina, I., Achen, M.G., Stacker, S.A., Hedman, M., Alitalo, K. and Yla-Herttuala, S., 2003. Angiogenic

responses of vascular endothelial growth factors in periadventitial tissue. Hum Gene Ther 14, 1451-62.

Bhardwaj, S., Roy, H., Heikura, T. and Yla-Herttuala, S., 2005. VEGF-A, VEGF-D and VEGF-D(DeltaNDeltaC) induced intimal hyperplasia in carotid arteries. Eur J Clin Invest 35, 669-76.

Bostrom, P., Wu, J., Jedrychowski, M.P., Korde, A., Ye, L., Lo, J.C., Rasbach, K.A., Bostrom, E.A., Choi, J.H., Long, J.Z., Kajimura, S., Zingaretti, M.C., Vind, B.F., Tu, H., Cinti, S., Hojlund, K., Gygi, S.P. and Spiegelman, B.M., 2012. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 481, 463-8.

Broxmeyer, H.E., Cooper, S., Li, Z.H., Lu, L., Song, H.Y., Kwon, B.S., Warren, R.E. and Donner, D.B., 1995. Myeloid progenitor cell regulatory effects of vascular endothelial cell growth factor. Int J Hematol 62, 203-15.

Bueno, O.F., De Windt, L.J., Tymitz, K.M., Witt, S.A., Kimball, T.R., Klevitsky, R., Hewett, T.E., Jones, S.P., Lefer, D.J., Peng, C.F., Kitsis, R.N. and Molkentin, J.D., 2000. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO J 19, 6341-50.

Butz, G.M. and Davisson, R.L., 2001. Long-term telemetric measurement of cardiovascular parameters in awake mice: a physiological genomics tool. Physiol Genomics 5, 89-97.

Cai, C.L., Martin, J.C., Sun, Y., Cui, L., Wang, L., Ouyang, K., Yang, L., Bu, L., Liang, X., Zhang, X., Stallcup, W.B., Denton, C.P., McCulloch, A., Chen, J. and Evans, S.M., 2008. A myocardial lineage derives from Tbx18 epicardial cells. Nature 454, 104-8.

Cao, Y., Ji, W.R., Qi, P., Rosin, A. and Cao, Y., 1997. Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. Biochem Biophys Res Commun 235, 493-8.

Cao, Y., Linden, P., Shima, D., Browne, F. and Folkman, J., 1996. In vivo angiogenic activity and hypoxia induction of heterodimers of placenta growth factor/vascular endothelial growth factor. J Clin Invest 98, 2507-11.

Capoccia, B.J., Huh, W.J. and Mills, J.C., 2009. How form follows functional genomics: gene expression profiling gastric epithelial cells with a particular discourse on the parietal cell. Physiol Genomics 37, 67-78.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Ebenhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A., 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380, 435-439.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H., Scholz, D., Acker, T., DiPalma, T., Dewerchin, M., Noel, A., Stalmans, I., Barra, A., Blacher, S., Vandendriessche, T., Ponten, A., Eriksson, U., Plate, K.H., Foidart, J.M., Schaper, W., Charnock-Jones, D.S., Hicklin, D.J., Herbert, J.M., Collen, D. and Persico, M.G., 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 7, 575-83.

Celletti, F.L., Waugh, J.M., Amabile, P.G., Brendolan, A., Hilfiker, P.R. and Dake, M.D., 2001. Vascular endothelial growth factor enhances atherosclerotic plaque progression. Nat Med 7, 425-429.

Chen, H., Chedotal, A., He, Z., Goodman, C.S. and Tessier-Lavigne, M., 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19, 547-559.

Christinger, H.W., Fuh, G., de Vos, A.M. and Wiesmann, C., 2004. The crystal structure of placental growth factor in complex with domain 2 of vascular endothelial growth factor receptor-1. J Biol Chem 279, 10382-8.

Chung, A.S. and Ferrara, N., 2011. Developmental and pathological angiogenesis. Annu Rev Cell Dev Biol 27, 563-84.

Clauss, M., Gerlach, M., Gerlach, H., Brett, J., Wang, F., Familetti, P.C., Pan, Y.-C.E., Olander, J.V., Connolly, D.T. and Stern, D., 1990. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity and promotes monocyte migration. J Exp Med 172, 1535-1545.

Clauss, M., Weich, H., Breier, G., Knies, U., Rockl, W., Waltenberger, J. and Risau, W., 1996. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. J Biol Chem 271, 17629-34.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons, L., Collen, D. and Carmeliet, P., 2002. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med 8, 702-10.

Condorelli, G., Drusco, A., Stassi, G., Bellacosa, A., Roncarati, R., Iaccarino, G., Russo, M.A., Gu, Y., Dalton, N., Chung, C., Latronico, M.V., Napoli, C., Sadoshima, J., Croce, C.M. and Ross, J., Jr., 2002. Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. Proc Natl Acad Sci U S A 99, 12333-8.

Cristofaro, B., Shi, Y., Faria, M., Suchting, S., Leroyer, A.S., Trindade, A., Duarte, A., Zovein, A.C., Iruela-Arispe, M.L., Nih, L.R., Kubis, N., Henrion, D., Loufrani, L., Todiras, M., Schleifenbaum, J., Gollasch, M., Zhuang, Z.W., Simons, M., Eichmann, A. and le Noble, F., 2013. Dll4-Notch signaling determines the formation of native arterial collateral networks and arterial function in mouse ischemia models. Development 140, 1720-9.

Cui, X., Ji, D., Fisher, D.A., Wu, Y., Briner, D.M. and Weinstein, E.J., 2011. Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 29, 64-7.

Davis-Smyth, T., Presta, L.G. and Ferrara, N., 1998. Mapping the charged residues in the second immunoglobulin-like domain of the vascular endothelial growth factor/placenta growth factor receptor Flt-1 required for binding and structural stability. J Biol Chem 273, 3216-22.

De Falco, S., 2012. The discovery of placenta growth factor and its biological activity. Exp Mol Med 44, 1-9.

De Vries, C., Escobedo, J.A., Ueno, H., Houck, K., Ferrara, N. and Williams, L.T., 1992. The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255, 989-991.

Deindl, E., Buschmann, I., Hoefer, I.E., Podzuweit, T., Boengler, K., Vogel, S., van Royen, N., Fernandez, B. and Schaper, W., 2001. Role of ischemia and of hypoxia-inducible genes in arteriogenesis after femoral artery occlusion in the rabbit. Circ Res 89, 779-86.

Detmar, M., Brown, L.F., Schon, M.P., Elicker, B.M., Velasco, P., Richard, L., Fukumura, D., Monsky, W., Claffey, K.P. and Jain, R.K., 1998. Increased

microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. J Invest Dermatol 111, 1-6.

Dettman, R.W., Denetclaw, W., Jr., Ordahl, C.P. and Bristow, J., 1998. Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. Dev Biol 193, 169-81.

Devaux, Y., Azuaje, F., Vausort, M., Yvorra, C. and Wagner, D.R., 2010. Integrated protein network and microarray analysis to identify potential biomarkers after myocardial infarction. Funct Integr Genomics 10, 329-37.

Devaux, Y., Vausort, M., Azuaje, F., Vaillant, M., Lair, M.L., Gayat, E., Lassus, J., Ng, L.L., Kelly, D., Wagner, D.R. and Squire, I.B., 2012. Low levels of vascular endothelial growth factor B predict left ventricular remodeling after acute myocardial infarction. J Card Fail 18, 330-7.

Dhondt, J., Peeraer, E., Verheyen, A., Nuydens, R., Buysschaert, I., Poesen, K., Van Geyte, K., Beerens, M., Shibuya, M., Haigh, J.J., Meert, T., Carmeliet, P. and Lambrechts, D., 2011. Neuronal FLT1 receptor and its selective ligand VEGF-B protect against retrograde degeneration of sensory neurons. FASEB J 25, 1461-73.

DiSalvo, J., Bayne, M.L., Conn, G., Kwok, P.W., Trivedi, P.G., Soderman, D.D., Palisi, T.M., Sullivan, K.A. and Thomas, K.A., 1995. Purification and characterization of a naturally occurring vascular endothelial growth factor.placenta growth factor heterodimer. Journal of Biological Chemistry 270, 7717-23.

Dixelius, J., Makinen, T., Wirzenius, M., Karkkainen, M.J., Wernstedt, C., Alitalo, K. and Claesson-Welsh, L., 2003. Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem 278, 40973-9.

Dorn, G.W., 2nd, 2007. The fuzzy logic of physiological cardiac hypertrophy. Hypertension 49, 962-70.

Dumont, D.J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M. and Alitalo, K., 1998. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 282, 946-9.

Duvekot, J.J. and Peeters, L.L., 1994. Maternal cardiovascular hemodynamic adaptation to pregnancy. Obstet Gynecol Surv 49, S1-14.

Ekman, N., Lymboussaki, A., Vastrik, I., Sarvas, K., Kaipainen, A. and Alitalo, K., 1997. Bmx tyrosine kinase is specifically expressed in the endocardium and the endothelium of large arteries. Circulation 96, 1729-32.

Enholm, B., Paavonen, K., Ristimaki, A., Kumar, V., Gunji, Y., Klefstrom, J., Kivinen, L., Laiho, M., Olofsson, B., Joukov, V., Eriksson, U. and Alitalo, K., 1997. Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. Oncogene 14, 2475-83.

Falk, T., Zhang, S. and Sherman, S.J., 2009. Vascular endothelial growth factor B (VEGF-B) is up-regulated and exogenous VEGF-B is neuroprotective in a culture model of Parkinson's disease. Mol Neurodegener 4, 49.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J. and Moore, M.W., 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380, 439-42.

Ferrara, N., Gerber, H.P. and LeCouter, J., 2003. The biology of VEGF and its receptors. Nat Med 9, 669-76.

Ferrara, N. and Henzel, W.J., 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161, 851-8.

Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., Chorianopoulos, E., Liesenborghs, L., Koch, M., De Mol, M., Autiero, M., Wyns, S., Plaisance, S., Moons, L., van Rooijen, N., Giacca, M., Stassen, J.M., Dewerchin, M., Collen, D. and Carmeliet, P., 2007. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell 131, 463-75.

Fong, G.H., Rossant, J., Gertsenstein, M. and Breitman, M.L., 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376, 66-70.

Fong, G.H., Zhang, L., Bryce, D.M. and Peng, J., 1999. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development 126, 3015-25.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D. and Semenza, G.L., 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol. Cell. Biol. 16, 4604-4613.

Freitas-Andrade, M., Carmeliet, P., Charlebois, C., Stanimirovic, D.B. and Moreno, M.J., 2012. PlGF knockout delays brain vessel growth and maturation upon systemic hypoxic challenge. J Cereb Blood Flow Metab 32, 663-75.

Fuh, G., Garcia, K.C. and de Vos, A.M., 2000. The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. J. Biol. Chem. 275, 26690-26695.

Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C.O., Buerk, D.G., Huang, P.L. and Jain, R.K., 2001. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Natl Acad Sci U S A 98, 2604-9.

Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A. and Sessa, W.C., 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399, 597-601.

Fulton, W.F., 1963a. Arterial Anastomoses in the Coronary Circulation. I. Anatomical Features in Normal and Diseased Hearts Demonstrated by Stereoarteriography. Scott Med J 8, 420-34.

Fulton, W.F., 1963b. Arterial Anastomoses in the Coronary Circulation. Ii. Distribution, Enumeration and Measurement of Coronary Arterial Anastomoses in Health and Disease. Scott Med J 8, 466-74.

Fulton, W.F., 1964. The Dynamic Factor in Enlargement of Coronary Arterial Anastomoses, and Paradoxical Changes in the Subendocardial Plexus. Br Heart J 26, 39-50.

Fulton, W.F.M. and van Royen, N., 2004. The Coronary Collateral Circulation in Man, in: Schaper, W. and Schaper, J. (Eds.), Arteriogenesis. Kluwer Academic Publishers, Boston ; London, pp. 297-331.

Gaal, E.I., Tammela, T., Anisimov, A., Marbacher, S., Honkanen, P., Zarkada, G., Leppanen, V.M., Tatlisumak, T., Hernesniemi, J., Niemela, M. and Alitalo, K., 2013. Comparison of vascular growth factors in the murine brain reveals placenta growth factor as prime candidate for CNS revascularization. Blood 122, 658-65.

Gagnon, M.L., Bielenberg, D.R., Gechtman, Z., Miao, H.Q., Takashima, S., Soker, S. and Klagsbrun, M., 2000. Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: In vivo expression and antitumor activity. Proc Natl Acad Sci U S A 97, 2573-8.

Gavard, J. and Gutkind, J.S., 2006. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol 8, 1223-34.

Gerace, L., Cirenei, N., Cappelletti, M., Petraroli, R., Sebastiani, F. and Marziliano, N., 2001. Assignment of the mouse Vegfb gene to mouse chromosome 19 B by in situ hybridization. Cytogenet Cell Genet 95, 242-3.

Gerber, H.P., Condorelli, F., Park, J. and Ferrara, N., 1997. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 272, 23659-67.

Gerber, H.P., Dixit, V. and Ferrara, N., 1998a. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 273, 13313-6.

Gerber, H.P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B.A., Dixit, V. and Ferrara, N., 1998b. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273, 30336-43.

Gibbs, R.A., Weinstock, G.M., Metzker, M.L., Muzny, D.M., Sodergren, E.J., Scherer, S., Scott, G., Steffen, D., Worley, K.C., Burch, P.E., Okwuonu, G., Hines, S., Lewis, L., DeRamo, C., Delgado, O., et al., 2004. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature 428, 493-521.

Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zioncheck, T.F., Pelletier, N. and Ferrara, N., 2001. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J. Biol. Chem. 276, 3222-3230.

Gille, H., Kowalski, J., Yu, L.L., Chen, H., Pisabarro, M.T., Davis-Smyth, T. and Ferrara, N., 2000. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3 '-kinase activation and endothelial cell migration. EMBO J. 19, 4064-4073.

Gluzman-Poltorak, Z., Cohen, T., Herzog, Y. and Neufeld, G., 2000. Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. J Biol Chem 275, 18040-5.

Gluzman-Poltorak, Z., Cohen, T., Shibuya, M. and Neufeld, G., 2001. Vascular endothelial growth factor receptor-1 and neuropilin-2 form complexes. J Biol Chem 276, 18688-94.

Grimmond, S., Lagercrantz, J., Drinkwater, C., Silins, G., Townson, S., Pollock, P., Gotley, D., Carson, E., Rakar, S., Nordenskjold, M., Ward, L., Hayward, N. and Weber, G., 1996. Cloning and characterization of a novel human gene related to vascular endothelial growth factor. Genome Res 6, 124-31.

Gross, L., 1921. The Blood Supply to the Heart, Oxford University Press.

Gunningham, S.P., Currie, M.J., Han, C., Turner, K., Scott, P.A., Robinson, B.A., Harris, A.L. and Fox, S.B., 2001. Vascular endothelial growth factor-B and vascular endothelial growth factor-C expression in renal cell carcinomas: regulation by the von Hippel-Lindau gene and hypoxia. Cancer Res 61, 3206-11.

Guo, D., Jia, Q., Song, H.Y., Warren, R.S. and Donner, D.B., 1995. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. J Biol Chem 270, 6729-33.

Hagberg, C.E., Falkevall, A., Wang, X., Larsson, E., Huusko, J., Nilsson, I., van Meeteren, L.A., Samen, E., Lu, L., Vanwildemeersch, M., Klar, J., Genove, G., Pietras, K., Stone-Elander, S., Claesson-Welsh, L., Yla-Herttuala, S., Lindahl, P. and Eriksson, U., 2010. Vascular endothelial growth factor B controls endothelial fatty acid uptake. Nature 464, 917-21.

Hagberg, C.E., Mehlem, A., Falkevall, A., Muhl, L., Fam, B.C., Ortsater, H., Scotney, P., Nyqvist, D., Samen, E., Lu, L., Stone-Elander, S., Proietto, J., Andrikopoulos, S., Sjoholm, A., Nash, A. and Eriksson, U., 2012. Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes. Nature 490, 426-30.

Haiko, P., Makinen, T., Keskitalo, S., Taipale, J., Karkkainen, M.J., Baldwin, M.E., Stacker, S.A., Achen, M.G. and Alitalo, K., 2008. Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. Mol Cell Biol 28, 4843-50.

Hamm, A., Veschini, L., Takeda, Y., Costa, S., Delamarre, E., Squadrito, M.L., Henze, A.T., Wenes, M., Serneels, J., Pucci, F., Roncal, C., Anisimov, A., Alitalo, K., De Palma, M. and Mazzone, M., 2013. PHD2 regulates arteriogenic macrophages through TIE2 signalling. EMBO Mol Med 5, 843-57.

Hanrahan, V., Currie, M.J., Gunningham, S.P., Morrin, H.R., Scott, P.A., Robinson, B.A. and Fox, S.B., 2003. The angiogenic switch for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in the adenoma-carcinoma sequence during colorectal cancer progression. J Pathol 200, 183-94.

Heberden, W., 1785. A letter to Dr. Heberden, concerning the angina pectoris; and an account of the dissection of one who had been troubled with that disorder. Read at the college November 17, 1772. Medical Transactions Published by the College of Physicians in London 3:1-11.

Heberlein, K.R., Straub, A.C., Best, A.K., Greyson, M.A., Looft-Wilson, R.C., Sharma, P.R., Meher, A., Leitinger, N. and Isakson, B.E., 2010. Plasminogen activator inhibitor-1 regulates myoendothelial junction formation. Circ Res 106, 1092-102.

Heil, M. and Schaper, W., 2004. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). Circ Res 95, 449-58.

Hershey, J.C., Baskin, E.P., Glass, J.D., Hartman, H.A., Gilberto, D.B., Rogers, I.T. and Cook, J.J., 2001. Revascularization in the rabbit hindlimb: dissociation between capillary sprouting and arteriogenesis. Cardiovasc Res 49, 618-25.

Hill, J.A. and Olson, E.N., 2008. Cardiac plasticity. N Engl J Med 358, 1370-80.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. and Shibuya, M., 1998. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proc Natl Acad Sci U S A 95, 9349-54.

Ho, V.C., Duan, L.J., Cronin, C., Liang, B.T. and Fong, G.H., 2012. Elevated VEGF Receptor-2 Abundance Contributes to Increased Angiogenesis in VEGF Receptor-1 Deficient Mice. Circulation.

Hoffman, J.I. and Buckberg, G.D., 1975. Pathophysiology of subendocardial ischaemia. Br Med J 1, 76-9.

Hunter, S. and Robson, S.C., 1992. Adaptation of the maternal heart in pregnancy. Br Heart J 68, 540-3.

Huusko, J., Lottonen, L., Merentie, M., Gurzeler, E., Anisimov, A., Miyanohara, A., Alitalo, K., Tavi, P. and Yla-Herttuala, S., 2012. AAV9-mediated VEGF-B gene transfer improves systolic function in progressive left ventricular hypertrophy. Mol Ther 20, 2212-21.

Huusko, J., Merentie, M., Dijkstra, M.H., Ryhanen, M.M., Karvinen, H., Rissanen, T.T., Vanwildemeersch, M., Hedman, M., Lipponen, J., Heinonen, S.E., Eriksson, U., Shibuya, M. and Yla-Herttuala, S., 2010. The effects of VEGF-R1 and VEGF-R2 ligands on angiogenic responses and left ventricular function in mice. Cardiovasc Res 86, 122-30.

Isner, J.M., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., Rosenfield, K., Razvi, S., Walsh, K. and Symes, J.F., 1996. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 348, 370-4.

Iyer, S., Darley, P.I. and Acharya, K.R., 2010. Structural insights into the binding of vascular endothelial growth factor-B by VEGFR-1(D2): recognition and specificity. J Biol Chem 285, 23779-89.

Iyer, S., Scotney, P.D., Nash, A.D. and Ravi Acharya, K., 2006. Crystal structure of human vascular endothelial growth factor-B: identification of amino acids important for receptor binding. J Mol Biol 359, 76-85.

Jaba, I.M., Zhuang, Z.W., Li, N., Jiang, Y., Martin, K.A., Sinusas, A.J., Papademetris, X., Simons, M., Sessa, W.C., Young, L.H. and Tirziu, D., 2013. NO triggers RGS4 degradation to coordinate angiogenesis and cardiomyocyte growth. J Clin Invest 123, 1718-31.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R.K. and Alitalo, K., 1997. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 276, 1423-1425.

Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N. and Alitalo, K., 1997. Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J. 16, 3898-3911.

Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V.W., Fang, G.H., Dumont, D., Breitman, M. and Alitalo, K., 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A 92, 3566-70.

Kaipainen, A., Korhonen, J., Pajusola, K., Aprelikova, O., Persico, M.G., Terman, B.I. and Alitalo, K., 1993. The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells. J Exp Med 178, 2077-88.

Kanasaki, K., Palmsten, K., Sugimoto, H., Ahmad, S., Hamano, Y., Xie, L., Parry, S., Augustin, H.G., Gattone, V.H., Folkman, J., Strauss, J.F. and Kalluri, R., 2008. Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. Nature 453, 1117-21.

Kantor, P.F., Lucien, A., Kozak, R. and Lopaschuk, G.D., 2000. The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-ketoacyl coenzyme A thiolase. Circ Res 86, 580-8.

Karkkainen, M.J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T.V., Jeltsch, M., Jackson, D.G., Talikka, M., Rauvala, H., Betsholtz, C. and Alitalo, K., 2004. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 5, 74-80.

Karkkainen, M.J., Saaristo, A., Jussila, L., Karila, K.A., Lawrence, E.C., Pajusola, K., Bueler, H., Eichmann, A., Kauppinen, R., Kettunen, M.I., Yla-Herttuala, S., Finegold, D.N., Ferrell, R.E. and Alitalo, K., 2001. A model for gene therapy of human hereditary lymphedema. Proc Natl Acad Sci U S A 98, 12677-82.

Karpanen, T., Heckman, C.A., Keskitalo, S., Jeltsch, M., Ollila, H., Neufeld, G., Tamagnone, L. and Alitalo, K., 2006. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. Faseb J 20, 1462-72.

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T. and Fujisawa, H., 1999. A requirement for neuropilin-1 in embryonic vessel formation. Development 126, 4895-902.

Kendall, R.L. and Thomas, K.A., 1993. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc. Natl. Acad. Sci. USA 90, 10705-10709.

Kenyon, B.M., Voest, E.E., Chen, C.C., Flynn, E., Folkman, J. and D'Amato, R.J., 1996. A model of angiogenesis in the mouse cornea. Invest Ophthalmol Vis Sci 37, 1625-32.

Kerkela, R., Woulfe, K. and Force, T., 2007. Glycogen synthase kinase-3beta -- actively inhibiting hypertrophy. Trends Cardiovasc Med 17, 91-6.

Khurana, R., Simons, M., Martin, J.F. and Zachary, I.C., 2005. Role of angiogenesis in cardiovascular disease: a critical appraisal. Circulation 112, 1813-24.

Kolakowski, S., Jr., Berry, M.F., Atluri, P., Grand, T., Fisher, O., Moise, M.A., Cohen, J., Hsu, V. and Woo, Y.J., 2006. Placental growth factor provides a novel local angiogenic therapy for ischemic cardiomyopathy. J Card Surg 21, 559-64.

Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J. and Ginty, D.D., 1997. Neuropilin is a semaphorin III receptor. Cell 90, 753-762.

Korff, T., Braun, J., Pfaff, D., Augustin, H.G. and Hecker, M., 2008. Role of ephrinB2 expression in endothelial cells during arteriogenesis: impact on smooth muscle cell migration and monocyte recruitment. Blood 112, 73-81.

Laakkonen, P., Waltari, M., Holopainen, T., Takahashi, T., Pytowski, B., Steiner, P., Hicklin, D., Persaud, K., Tonra, J.R., Witte, L. and Alitalo, K., 2007. Vascular endothelial growth factor receptor 3 is involved in tumor angiogenesis and growth. Cancer Res 67, 593-9.

Lagercrantz, J., Farnebo, F., Larsson, C., Tvrdik, T., Weber, G. and Piehl, F., 1998. A comparative study of the expression patterns for vegf, vegf-b/vrf and vegf-c in the developing and adult mouse. Biochim Biophys Acta 1398, 157-63.

Lahteenvuo, J.E., Lahteenvuo, M.T., Kivela, A., Rosenlew, C., Falkevall, A., Klar, J., Heikura, T., Rissanen, T.T., Vahakangas, E., Korpisalo, P., Enholm, B., Carmeliet, P., Alitalo, K., Eriksson, U. and Yla-Herttuala, S., 2009. Vascular endothelial growth factor-B induces myocardium-specific angiogenesis and arteriogenesis via vascular endothelial growth factor receptor-1- and neuropilin receptor-1-dependent mechanisms. Circulation 119, 845-56.

Landgren, E., Schiller, P., Cao, Y. and Claesson-Welsh, L., 1998. Placenta growth factor stimulates MAP kinase and mitogenicity but not phospholipase C- gamma and migration of endothelial cells expressing Flt 1. Oncogene 16, 359-367.

Lanza, G.A. and Crea, F., 2010. Primary coronary microvascular dysfunction: clinical presentation, pathophysiology, and management. Circulation 121, 2317-25.

Larcher, F., Murillas, R., Bolontrade, M., Conti, C.J. and Jorcano, J.L., 1998. VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and accelerated tumor development. Oncogene 17, 303-11.

Lee, R.J., Springer, M.L., Blanco-Bose, W.E., Shaw, R., Ursell, P.C. and Blau, H.M., 2000. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation 102, 898-901.

Leppanen, P., Koota, S., Kholova, I., Koponen, J., Fieber, C., Eriksson, U., Alitalo, K. and Yla-Herttuala, S., 2005. Gene transfers of vascular endothelial growth factor-A, vascular endothelial growth factor-B, vascular endothelial growth factor-C, and vascular endothelial growth factor-D have no effects on atherosclerosis in hypercholesterolemic low-density lipoprotein-receptor/apolipoprotein B48-deficient mice. Circulation 112, 1347-52.

Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V. and Ferrara, N., 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246, 1306-9.

Levine, R.J., Maynard, S.E., Qian, C., Lim, K.H., England, L.J., Yu, K.F., Schisterman, E.F., Thadhani, R., Sachs, B.P., Epstein, F.H., Sibai, B.M., Sukhatme, V.P. and Karumanchi, S.A., 2004. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 350, 672-83.

Li, X., Aase, K., Li, H., von Euler, G. and Eriksson, U., 2001. Isoform-specific expression of VEGF-B in normal tissues and tumors. Growth Factors 19, 49-59.

Li, X., Tjwa, M., Van Hove, I., Enholm, B., Neven, E., Paavonen, K., Jeltsch, M., Juan, T.D., Sievers, R.E., Chorianopoulos, E., Wada, H., Vanwildemeersch, M., Noel, A., Foidart, J.M., Springer, M.L., von Degenfeld, G., Dewerchin, M., Blau, H.M., Alitalo, K., Eriksson, U., Carmeliet, P. and Moons, L., 2008a. Reevaluation of the role of VEGF-B suggests a restricted role in the revascularization of the ischemic myocardium. Arterioscler Thromb Vasc Biol 28, 1614-20.

Li, Y., Zhang, F., Nagai, N., Tang, Z., Zhang, S., Scotney, P., Lennartsson, J., Zhu, C., Qu, Y., Fang, C., Hua, J., Matsuo, O., Fong, G.H., Ding, H., Cao, Y., Becker, K.G., Nash, A., Heldin, C.H. and Li, X., 2008b. VEGF-B inhibits apoptosis via VEGFR-1-mediated suppression of the expression of BH3-only protein genes in mice and rats. J Clin Invest 118, 913-23.

Limbourg, A., Ploom, M., Elligsen, D., Sorensen, I., Ziegelhoeffer, T., Gossler, A., Drexler, H. and Limbourg, F.P., 2007. Notch ligand Delta-like 1 is essential for postnatal arteriogenesis. Circ Res 100, 363-71.

Liu, X., Claus, P., Wu, M., Reyns, G., Verhamme, P., Pokreisz, P., Vandenwijngaert, S., Dubois, C., Vanhaecke, J., Verbeken, E., Bogaert, J. and Janssens, S., 2013. Placental growth factor increases regional myocardial blood flow and contractile function in chronic myocardial ischemia. Am J Physiol Heart Circ Physiol 304, H885-94.

Liu, Z.J., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G.P., Fairman, R.M., Velazquez, O.C. and Herlyn, M., 2003. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. Mol Cell Biol 23, 14-25.

Lohela, M., Bry, M., Tammela, T. and Alitalo, K., 2009. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. Curr Opin Cell Biol 21, 154-65.

Lower, R., 1669. Tractatus de corde, item de motu et colore sanguinis, et chyli in eum transitu.

Lumens, J., Delhaas, T., Arts, T., Cowan, B.R. and Young, A.A., 2006. Impaired subendocardial contractile myofiber function in asymptomatic aged humans, as detected using MRI. Am J Physiol Heart Circ Physiol 291, H1573-9.

Luttun, A., Tjwa, M., Moons, L., Wu, Y., Angelillo-Scherrer, A., Liao, F., Nagy, J.A., Hooper, A., Priller, J., De Klerck, B., Compernolle, V., Daci, E., Bohlen, P., Dewerchin, M., Herbert, J.M., Fava, R., Matthys, P., Carmeliet, G., Collen, D., Dvorak, H.F., Hicklin, D.J. and Carmeliet, P., 2002. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med 8, 831-40.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P. and Persico, M.G., 1991. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci U S A 88, 9267-71.

Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M.G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K.-J., Chou, J.Y. and Persico, M.G., 1993. Two alternative

mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene 8, 925-931.

Maillet, M., van Berlo, J.H. and Molkentin, J.D., 2013. Molecular basis of physiological heart growth: fundamental concepts and new players. Nat Rev Mol Cell Biol 14, 38-48.

Makinen, T., Olofsson, B., Karpanen, T., Hellman, U., Soker, S., Klagsbrun, M., Eriksson, U. and Alitalo, K., 1999. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. J Biol Chem 274, 21217-22.

Maron, B.J. and Pelliccia, A., 2006. The heart of trained athletes: cardiac remodeling and the risks of sports, including sudden death. Circulation 114, 1633-44.

Marzilli, M., Merz, C.N., Boden, W.E., Bonow, R.O., Capozza, P.G., Chilian, W.M., DeMaria, A.N., Guarini, G., Huqi, A., Morrone, D., Patel, M.R. and Weintraub, W.S., 2012. Obstructive coronary atherosclerosis and ischemic heart disease: an elusive link! J Am Coll Cardiol 60, 951-6.

Mees, B., Wagner, S., Ninci, E., Tribulova, S., Martin, S., van Haperen, R., Kostin, S., Heil, M., de Crom, R. and Schaper, W., 2007. Endothelial nitric oxide synthase activity is essential for vasodilation during blood flow recovery but not for arteriogenesis. Arterioscler Thromb Vasc Biol 27, 1926-33.

Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H.G., Ziche, M., Lanz, C., Buttner, M., Rziha, H.J. and Dehio, C., 1999. A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. Embo J 18, 363-74.

Michaels, L., 1966. Aetiology of coronary artery disease: an historical approach. Br Heart J 28, 258-64.

Migdal, M., Huppertz, B., Tessler, S., Comforti, A., Shibuya, M., Reich, R., Baumann, H. and Neufeld, G., 1998. Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem 273, 22272-22278.

Mikawa, T. and Gourdie, R.G., 1996. Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. Dev Biol 174, 221-32.

Mitchell-Jordan, S.A., Holopainen, T., Ren, S., Wang, S., Warburton, S., Zhang, M.J., Alitalo, K., Wang, Y. and Vondriska, T.M., 2008. Loss of Bmx nonreceptor tyrosine kinase prevents pressure overload-induced cardiac hypertrophy. Circ Res 103, 1359-62.

Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R. and Olson, E.N., 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93, 215-28.

Mootha, V.K., Bunkenborg, J., Olsen, J.V., Hjerrild, M., Wisniewski, J.R., Stahl, E., Bolouri, M.S., Ray, H.N., Sihag, S., Kamal, M., Patterson, N., Lander, E.S. and Mann, M., 2003. Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. Cell 115, 629-40.

Mould, A.W., Greco, S.A., Cahill, M.M., Tonks, I.D., Bellomo, D., Patterson, C., Zournazi, A., Nash, A., Scotney, P., Hayward, N.K. and Kay, G.F., 2005. Transgenic overexpression of vascular endothelial growth factor-B isoforms by endothelial cells potentiates postnatal vessel growth in vivo and in vitro. Circ Res 97, e60-70.

Mould, A.W., Tonks, I.D., Cahill, M.M., Pettit, A.R., Thomas, R., Hayward, N.K. and Kay, G.F., 2003. Vegfb gene knockout mice display reduced pathology and synovial

angiogenesis in both antigen-induced and collagen-induced models of arthritis. Arthritis Rheum 48, 2660-9.

Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., Huang, P.L. and Isner, J.M., 1998. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest 101, 2567-78.

Nagy, J.A., Benjamin, L., Zeng, H., Dvorak, A.M. and Dvorak, H.F., 2008. Vascular permeability, vascular hyperpermeability and angiogenesis. Angiogenesis 11, 109-19.

Nilsson, I., Bahram, F., Li, X., Gualandi, L., Koch, S., Jarvius, M., Soderberg, O., Anisimov, A., Kholova, I., Pytowski, B., Baldwin, M., Yla-Herttuala, S., Alitalo, K., Kreuger, J. and Claesson-Welsh, L., 2010. VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. EMBO J 29, 1377-88.

Nucera, S., Biziato, D. and De Palma, M., 2011. The interplay between macrophages and angiogenesis in development, tissue injury and regeneration. Int J Dev Biol 55, 495-503.

Odorisio, T., Schietroma, C., Zaccaria, M.L., Cianfarani, F., Tiveron, C., Tatangelo, L., Failla, C.M. and Zambruno, G., 2002. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. J Cell Sci 115, 2559-67.

Olofsson, B., Korpelainen, E., Pepper, M.S., Mandriota, S.J., Aase, K., Kumar, V., Gunji, Y., Jeltsch, M.M., Shibuya, M., Alitalo, K. and Eriksson, U., 1998a. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci U S A 95, 11709-14.

Olofsson, B., Korpelainen, E., Pepper, M.S., Mandriota, S.J., Aase, K., Kumar, V., Gunji, Y., Jeltsch, M.M., Shibuya, M., Alitalo, K. and Eriksson, U., 1998b. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc. Natl. Acad. Sci. USA 95, 11709-11714.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R.F., Alitalo, K. and Eriksson, U., 1996a. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc. Natl Acad. Sci. USA 93, 2576-2581.

Olofsson, B., Pajusola, K., von Euler, G., Chilov, D., Alitalo, K. and Eriksson, U., 1996b. Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. J. Biol. Chem. 271, 19310-19317.

Olsson, A.K., Dimberg, A., Kreuger, J. and Claesson-Welsh, L., 2006. VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 7, 359-71.

Oura, H., Bertoncini, J., Velasco, P., Brown, L.F., Carmeliet, P. and Detmar, M., 2003. A critical role of placental growth factor in the induction of inflammation and edema formation. Blood 101, 560-7.

Paavonen, K., Horelli-Kuitunen, N., Chilov, D., Kukk, E., Pennanen, S., Kallioniemi, O.P., Pajusola, K., Olofsson, B., Eriksson, U., Joukov, V., Palotie, A. and Alitalo, K., 1996. Novel human vascular endothelial growth factor genes VEGF-B and VEGF-C localize to chromosomes 11q13 and 4q34, respectively. Circulation 93, 1079-82.

Park, J.E., Chen, H.H., Winer, J., Houck, K.A. and Ferrara, N., 1994. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 269, 25646-54.

Partanen, T.A., Arola, J., Saaristo, A., Jussila, L., Ora, A., Miettinen, M., Stacker, S.A., Achen, M.G. and Alitalo, K., 2000. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. Faseb J 14, 2087-96.

Partanen, T.A., Makinen, T., Arola, J., Suda, T., Weich, H.A. and Alitalo, K., 1999. Endothelial growth factor receptors in human fetal heart. Circulation 100, 583-586.

Passier, R., Zeng, H., Frey, N., Naya, F.J., Nicol, R.L., McKinsey, T.A., Overbeek, P., Richardson, J.A., Grant, S.R. and Olson, E.N., 2000. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J Clin Invest 105, 1395-406.

Patila, T., Ikonen, T., Rutanen, J., Ahonen, A., Lommi, J., Lappalainen, K., Krogerus, L., Ihlberg, L., Partanen, T.A., Lahteenoja, L., Virtanen, K., Alitalo, K., Yla-Herttuala, S. and Harjula, A., 2006. Vascular endothelial growth factor C-induced collateral formation in a model of myocardial ischemia. J Heart Lung Transplant 25, 206-13.

Patrucco, E., Notte, A., Barberis, L., Selvetella, G., Maffei, A., Brancaccio, M., Marengo, S., Russo, G., Azzolino, O., Rybalkin, S.D., Silengo, L., Altruda, F., Wetzker, R., Wymann, M.P., Lembo, G. and Hirsch, E., 2004. PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. Cell 118, 375-87.

Pellet-Many, C., Frankel, P., Jia, H. and Zachary, I., 2008. Neuropilins: structure, function and role in disease. Biochem J 411, 211-26.

Pelliccia, A., Culasso, F., Di Paolo, F.M. and Maron, B.J., 1999. Physiologic left ventricular cavity dilatation in elite athletes. Ann Intern Med 130, 23-31.

Pelliccia, A., Maron, B.J., De Luca, R., Di Paolo, F.M., Spataro, A. and Culasso, F., 2002. Remodeling of left ventricular hypertrophy in elite athletes after long-term deconditioning. Circulation 105, 944-9.

Pepe, M., Mamdani, M., Zentilin, L., Csiszar, A., Qanud, K., Zacchigna, S., Ungvari, Z., Puligadda, U., Moimas, S., Xu, X., Edwards, J.G., Hintze, T.H., Giacca, M. and Recchia, F.A., 2010. Intramyocardial VEGF-B167 gene delivery delays the progression towards congestive failure in dogs with pacing-induced dilated cardiomyopathy. Circ Res 106, 1893-903.

Pepper, M.S., 2001. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 21, 1104-17.

Pepper, M.S., Ferrara, N., Orci, L. and Montesano, R., 1991. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem Biophys Res Commun 181, 902-6.

Perez-Pomares, J.M., Carmona, R., Gonzalez-Iriarte, M., Atencia, G., Wessels, A. and Munoz-Chapuli, R., 2002. Origin of coronary endothelial cells from epicardial mesothelium in avian embryos. Int J Dev Biol 46, 1005-13.

Pettersson, A., Nagy, J.A., Brown, L.F., Sundberg, C., Morgan, E., Jungles, S., Carter, R., Krieger, J.E., Manseau, E.J., Harvey, V.S., Eckelhoefer, I.A., Feng, D., Dvorak, A.M., Mulligan, R.C. and Dvorak, H.F., 2000. Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. Lab Invest 80, 99-115.

Pfeffer, M.A., Pfeffer, J.M., Fishbein, M.C., Fletcher, P.J., Spadaro, J., Kloner, R.A. and Braunwald, E., 1979. Myocardial infarct size and ventricular function in rats. Circ Res 44, 503-12.

Pipp, F., Heil, M., Issbrucker, K., Ziegelhoeffer, T., Martin, S., van den Heuvel, J., Weich, H., Fernandez, B., Golomb, G., Carmeliet, P., Schaper, W. and Clauss, M., 2003. VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. Circ Res 92, 378-85.

Poesen, K., Lambrechts, D., Van Damme, P., Dhondt, J., Bender, F., Frank, N., Bogaert, E., Claes, B., Heylen, L., Verheyen, A., Raes, K., Tjwa, M., Eriksson, U., Shibuya, M., Nuydens, R., Van Den Bosch, L., Meert, T., D'Hooge, R., Sendtner, M., Robberecht, W. and Carmeliet, P., 2008. Novel role for vascular endothelial growth factor (VEGF) receptor-1 and its ligand VEGF-B in motor neuron degeneration. J Neurosci 28, 10451-9.

Polverini, P.J., Cotran, P.S., Gimbrone, M.A., Jr. and Unanue, E.R., 1977. Activated macrophages induce vascular proliferation. Nature 269, 804-6.

Proudfit, W.L., 1983. Origin of concept of ischaemic heart disease. Br Heart J 50, 209-12.

Pugh, C.W. and Ratcliffe, P.J., 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9, 677-84.

Quinn, T., Peters, K., DeVries, C., Ferrara, N. and Williams, L., 1993. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc.Natl.Acad.Sci.USA 90, 7533-7537.

Rajantie, I., Ekman, N., Iljin, K., Arighi, E., Gunji, Y., Kaukonen, J., Palotie, A., Dewerchin, M., Carmeliet, P. and Alitalo, K., 2001. Bmx tyrosine kinase has a redundant function downstream of angiopoietin and vascular endothelial growth factor receptors in arterial endothelium. Mol Cell Biol 21, 4647-55.

Red-Horse, K., Ueno, H., Weissman, I.L. and Krasnow, M.A., 2010. Coronary arteries form by developmental reprogramming of venous cells. Nature 464, 549-53.

Risau, W., 1997. Mechanisms of angiogenesis. Nature 386, 671-4.

Risau, W. and Flamme, I., 1995. Vasculogenesis. Annu Rev Cell Dev Biol 11, 73-91.

Rissanen, T.T., Markkanen, J.E., Gruchala, M., Heikura, T., Puranen, A., Kettunen, M.I., Kholova, I., Kauppinen, R.A., Achen, M.G., Stacker, S.A., Alitalo, K. and Yla-Herttuala, S., 2003. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res 92, 1098-106.

Robinson, C.J. and Stringer, S.E., 2001. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 114, 853-65.

Rossignol, M., Gagnon, M.L. and Klagsbrun, M., 2000. Genomic organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. Genomics 70, 211-22.

Ruiz de Almodovar, C., Lambrechts, D., Mazzone, M. and Carmeliet, P., 2009. Role and therapeutic potential of VEGF in the nervous system. Physiol Rev 89, 607-48.

Rutanen, J., Rissanen, T.T., Markkanen, J.E., Gruchala, M., Silvennoinen, P., Kivela, A., Hedman, A., Hedman, M., Heikura, T., Orden, M.R., Stacker, S.A., Achen, M.G., Hartikainen, J. and Yla-Herttuala, S., 2004. Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of vascular endothelial growth factor-D induces transmural angiogenesis in porcine heart. Circulation 109, 1029-35.

Saaristo, A., Veikkola, T., Enholm, B., Hytonen, M., Arola, J., Pajusola, K., Turunen, P., Jeltsch, M., Karkkainen, M.J., Kerjaschki, D., Bueler, H., Yla-Herttuala, S. and Alitalo, K., 2002. Adenoviral VEGF-C overexpression induces blood vessel enlargement, tortuosity, and leakiness but no sprouting angiogenesis in the skin or mucous membranes. Faseb J 16, 1041-9.

Sadler, T.W., 2006. Langman's medical embryology, 10th ed. Lippincott Williams & Wilkins, Philadelphia.

Saharinen, P., Bry, M. and Alitalo, K., 2010. How do angiopoietins Tie in with vascular endothelial growth factors? Curr Opin Hematol 17, 198-205.

Sano, M., Minamino, T., Toko, H., Miyauchi, H., Orimo, M., Qin, Y., Akazawa, H., Tateno, K., Kayama, Y., Harada, M., Shimizu, I., Asahara, T., Hamada, H., Tomita, S., Molkentin, J.D., Zou, Y. and Komuro, I., 2007. p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. Nature 446, 444-8.

Schannwell, C.M., Zimmermann, T., Schneppenheim, M., Plehn, G., Marx, R. and Strauer, B.E., 2002. Left ventricular hypertrophy and diastolic dysfunction in healthy pregnant women. Cardiology 97, 73-8.

Schaper, W., 2009. Collateral circulation: past and present. Basic Res Cardiol 104, 5-21.

Schaper, W., Piek, J.J., Munoz-Chapuli, R., Wolf, C. and Ito, W., 1999. Collateral circulation of the heart, in: Ware, J.A. and Simons, M. (Eds.), Angiogenesis and Cardiovascular Disease. Oxford University Press, New York, NY ; Oxford, pp. 159-198.

Schaper, W. and Schaper, J., 2004. Arteriogenesis, Kluwer Academic Publishers, Boston ; London.

Schoenfeld, J., Lessan, K., Johnson, N.A., Charnock-Jones, D.S., Evans, A., Vourvouhaki, E., Scott, L., Stephens, R., Freeman, T.C., Saidi, S.A., Tom, B., Weston, G.C., Rogers, P., Smith, S.K. and Print, C.G., 2004. Bioinformatic analysis of primary endothelial cell gene array data illustrated by the analysis of transcriptome changes in endothelial cells exposed to VEGF-A and PlGF. Angiogenesis 7, 143-56.

Schweiki, D., Itin, A., Soffer, D. and Keshet, E., 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359, 843-848.

Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S. and Shibuya, M., 1995. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene 10, 135-47.

Seiler, C., 2009. Collateral circulation of the heart, Springer, New York ; London.

Selvaraj, S.K., Giri, R.K., Perelman, N., Johnson, C., Malik, P. and Kalra, V.K., 2003. Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor. Blood 102, 1515-24.

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S. and Dvorak, H.F., 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219, 983-5.

Serpi, R., Tolonen, A.M., Huusko, J., Rysa, J., Tenhunen, O., Yla-Herttuala, S. and Ruskoaho, H., 2011. Vascular endothelial growth factor-B gene transfer prevents angiotensin II-induced diastolic dysfunction via proliferation and capillary dilatation in rats. Cardiovasc Res 89, 204-13.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L. and Schuh, A.C., 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376, 62-6.

Sheikh, A.Y., Chun, H.J., Glassford, A.J., Kundu, R.K., Kutschka, I., Ardigo, D., Hendry, S.L., Wagner, R.A., Chen, M.M., Ali, Z.A., Yue, P., Huynh, D.T., Connolly, A.J., Pelletier, M.P., Tsao, P.S., Robbins, R.C. and Quertermous, T., 2008. In vivo genetic profiling and cellular localization of apelin reveals a hypoxia-sensitive, endothelial-centered pathway activated in ischemic heart failure. Am J Physiol Heart Circ Physiol 294, H88-98.

Shibuya, M., 2006. Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. Angiogenesis 9, 225-30; discussion 231.

Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H. and Sato, M., 1990. Nucleotide sequence and expression of a novel human receptor type tyrosine kinase gene (*flt*) closely related to the *fms* family. Oncogene 5, 519-524.

Shioi, T., Kang, P.M., Douglas, P.S., Hampe, J., Yballe, C.M., Lawitts, J., Cantley, L.C. and Izumo, S., 2000. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. EMBO J 19, 2537-48.

Shioi, T., McMullen, J.R., Tarnavski, O., Converso, K., Sherwood, M.C., Manning, W.J. and Izumo, S., 2003. Rapamycin attenuates load-induced cardiac hypertrophy in mice. Circulation 107, 1664-70.

Shiojima, I., Sato, K., Izumiya, Y., Schiekofer, S., Ito, M., Liao, R., Colucci, W.S. and Walsh, K., 2005. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. J Clin Invest 115, 2108-18.

Silvestre, J.S., Tamarat, R., Ebrahimian, T.G., Le-Roux, A., Clergue, M., Emmanuel, F., Duriez, M., Schwartz, B., Branellec, D. and Levy, B.I., 2003. Vascular endothelial growth factor-B promotes in vivo angiogenesis. Circ Res 93, 114-23.

Singh, N.K., Hansen, D.E., 3rd, Kundumani-Sridharan, V. and Rao, G.N., 2013. Both Kdr and Flt1 play a vital role in hypoxia-induced Src-PLD1-PKCgamma-cPLA(2) activation and retinal neovascularization. Blood 121, 1911-23.

Soker, S., Miao, H.Q., Nomi, M., Takashima, S. and Klagsbrun, M., 2002. VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. J Cell Biochem 85, 357-68.

Soker, S., Takashima, S., Miao, H.Q., Neufeld, G. and Klagsbrun, M., 1998. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92, 735-745.

Spalteholz, W., 1907. Die Koronararterien des Herzens, Verhandl Anat Gesell. pp. 141.

Sun, Y., Jin, K., Childs, J.T., Xie, L., Mao, X.O. and Greenberg, D.A., 2004. Increased severity of cerebral ischemic injury in vascular endothelial growth factor-B-deficient mice. J Cereb Blood Flow Metab 24, 1146-52.

Taegtmeyer, H., 2002. Switching metabolic genes to build a better heart. Circulation 106, 2043-5.

Taegtmeyer, H., 2007. Fueling the Heart: Multiple Roles for Cardiac Metabolism, in: Willerson, J.T., Cohn, J.N., Wellens, H.J. and Holmes, D.R. (Eds.), Cardiovascular Medicine. Springer, London, pp. 1157-1176.

Takahashi, T., Ueno, H. and Shibuya, M., 1999. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 18, 2221-30.

Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki Ji, J., Hirota, S., Kitamura, Y., Kitsukawa, T., Fujisawa, H., Klagsbrun, M. and Hori, M., 2002. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proc Natl Acad Sci U S A 99, 3657-62.

Takeda, Y., Costa, S., Delamarre, E., Roncal, C., Leite de Oliveira, R., Squadrito, M.L., Finisguerra, V., Deschoemaeker, S., Bruyere, F., Wenes, M., Hamm, A., Serneels, J., Magat, J., Bhattacharyya, T., Anisimov, A., Jordan, B.F., Alitalo, K., Maxwell, P., Gallez, B., Zhuang, Z.W., Saito, Y., Simons, M., De Palma, M. and Mazzone, M., 2011. Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. Nature 479, 122-6.

Tammela, T. and Alitalo, K., 2010. Lymphangiogenesis: Molecular mechanisms and future promise. Cell 140, 460-76.

Tammela, T., Zarkada, G., Nurmi, H., Jakobsson, L., Heinolainen, K., Tvorogov, D., Zheng, W., Franco, C.A., Murtomaki, A., Aranda, E., Miura, N., Yla-Herttuala, S., Fruttiger, M., Makinen, T., Eichmann, A., Pollard, J.W., Gerhardt, H. and Alitalo, K., 2011. VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. Nat Cell Biol 13, 1202-13.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Peltonen, R., Freitas, C., Duarte, A., Isoniemi, H., Laakkonen, P., Christofori, G., Yla-Herttuala, S., Shibuya, M., Pytowski, B., Eichmann, A., Betsholtz, C. and Alitalo, K., 2008. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature 454, 656-60.

Tanaka, K., Yamaguchi, S., Sawano, A. and Shibuya, M., 1997. Characterization of the extracellular domain in vascular endothelial growth factor receptor-1 (Flt-1 tyrosine kinase). Jpn J Cancer Res 88, 867-76.

Tirziu, D., Chorianopoulos, E., Moodie, K.L., Palac, R.T., Zhuang, Z.W., Tjwa, M., Roncal, C., Eriksson, U., Fu, Q., Elfenbein, A., Hall, A.E., Carmeliet, P., Moons, L. and Simons, M., 2007. Myocardial hypertrophy in the absence of external stimuli is induced by angiogenesis in mice. J Clin Invest 117, 3188-97.

Tirziu, D., Moodie, K.L., Zhuang, Z.W., Singer, K., Helisch, A., Dunn, J.F., Li, W., Singh, J. and Simons, M., 2005. Delayed arteriogenesis in hypercholesterolemic mice. Circulation 112, 2501-9.

Tomanek, R.J., Holifield, J.S., Reiter, R.S., Sandra, A. and Lin, J.J., 2002. Role of VEGF family members and receptors in coronary vessel formation. Dev Dyn 225, 233-40.

Tomanek, R.J., Ishii, Y., Holifield, J.S., Sjogren, C.L., Hansen, H.K. and Mikawa, T., 2006. VEGF family members regulate myocardial tubulogenesis and coronary artery formation in the embryo. Circ Res 98, 947-53.

Troidl, K., Tribulova, S., Cai, W.J., Ruding, I., Apfelbeck, H., Schierling, W., Troidl, C., Schmitz-Rixen, T. and Schaper, W., 2010. Effects of endogenous nitric oxide and of DETA NONOate in arteriogenesis. J Cardiovasc Pharmacol 55, 153-60.

van den Akker, N.M., Caolo, V., Wisse, L.J., Peters, P.P., Poelmann, R.E., Carmeliet, P., Molin, D.G. and Gittenberger-de Groot, A.C., 2008. Developmental coronary maturation is disturbed by aberrant cardiac vascular endothelial growth factor expression and Notch signalling. Cardiovasc Res 78, 366-75.

van Royen, N. and Schaper, W., 2004. Arteriogenesis and Atherosclerosis: Strange Bedfellows, in: Schaper, W. and Schaper, J. (Eds.), Arteriogenesis. Kluwer Academic Publishers, Boston ; London, pp. 361-369.

Vassar, R., Rosenberg, M., Ross, S., Tyner, A. and Fuchs, E., 1989. Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. Proc. Natl. Acad. Sci. USA 86, 1563-1567.

Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T.V., Kubo, H., Thurston, G., McDonald, D.M., Achen, M.G., Stacker, S.A. and Alitalo, K., 2001. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. Embo J 20, 1223-31.

Viragh, S. and Challice, C.E., 1981. The origin of the epicardium and the embryonic myocardial circulation in the mouse. Anat Rec 201, 157-68.

Vrancken Peeters, M.P., Gittenberger-de Groot, A.C., Mentink, M.M. and Poelmann, R.E., 1999. Smooth muscle cells and fibroblasts of the coronary arteries derive from

epithelial-mesenchymal transformation of the epicardium. Anat Embryol (Berl) 199, 367-78.
Wafai, R., Tudor, E.M., Angus, J.A. and Wright, C.E., 2009. Vascular effects of FGF-2 and VEGF-B in rabbits with bilateral hind limb ischemia. J Vasc Res 46, 45-54.
Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C.H., 1994. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J. Biol. Chem. 269, 26988-26995.
Wang, H.U., Chen, Z.F. and Anderson, D.J., 1998. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93, 741-53.
Wanstall, J.C., Gambino, A., Jeffery, T.K., Cahill, M.M., Bellomo, D., Hayward, N.K. and Kay, G.F., 2002. Vascular endothelial growth factor-B-deficient mice show impaired development of hypoxic pulmonary hypertension. Cardiovasc Res 55, 361-8.
Wilkins, B.J. and Molkentin, J.D., 2004. Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Biochem Biophys Res Commun 322, 1178-91.
Winter, E.M. and Gittenberger-de Groot, A.C., 2007. Epicardium-derived cells in cardiogenesis and cardiac regeneration. Cell Mol Life Sci 64, 692-703.
World Health Organization, 2008. The global burden of disease : 2004 update, World Health Organization, Geneva.
Xia, P., Aiello, L.P., Ishii, H., Jiang, Z.Y., Park, D.J., Robinson, G.S., Takagi, H., Newsome, W.P., Jirousek, M.R. and King, G.L., 1996. Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. J Clin Invest 98, 2018-26.
Xia, Y.P., Li, B., Hylton, D., Detmar, M., Yancopoulos, G.D. and Rudge, J.S., 2003. Transgenic delivery of VEGF to mouse skin leads to an inflammatory condition resembling human psoriasis. Blood 102, 161-8.
Yang, W., Ahn, H., Hinrichs, M., Torry, R.J. and Torry, D.S., 2003. Evidence of a novel isoform of placenta growth factor (PlGF-4) expressed in human trophoblast and endothelial cells. J Reprod Immunol 60, 53-60.
Yao, J., Wu, X., Zhuang, G., Kasman, I.M., Vogt, T., Phan, V., Shibuya, M., Ferrara, N. and Bais, C., 2011. Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy. Proc Natl Acad Sci U S A 108, 11590-5.
Yla-Herttuala, S., 2013. Cardiovascular gene therapy with vascular endothelial growth factors. Gene 525, 217-9.
Yla-Herttuala, S., Rissanen, T.T., Vajanto, I. and Hartikainen, J., 2007. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 49, 1015-26.
Young, M.E., Laws, F.A., Goodwin, G.W. and Taegtmeyer, H., 2001. Reactivation of peroxisome proliferator-activated receptor alpha is associated with contractile dysfunction in hypertrophied rat heart. J Biol Chem 276, 44390-5.
Yu, J., deMuinck, E.D., Zhuang, Z., Drinane, M., Kauser, K., Rubanyi, G.M., Qian, H.S., Murata, T., Escalante, B. and Sessa, W.C., 2005. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. Proc Natl Acad Sci U S A 102, 10999-1004.
Yuan, L., Moyon, D., Pardanaud, L., Breant, C., Karkkainen, M.J., Alitalo, K. and Eichmann, A., 2002. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development 129, 4797-806.

Zaha, V.G. and Young, L.H., 2012. AMP-activated protein kinase regulation and biological actions in the heart. Circ Res 111, 800-14.

Zentilin, L., Puligadda, U., Lionetti, V., Zacchigna, S., Collesi, C., Pattarini, L., Ruozi, G., Camporesi, S., Sinagra, G., Pepe, M., Recchia, F.A. and Giacca, M., 2010. Cardiomyocyte VEGFR-1 activation by VEGF-B induces compensatory hypertrophy and preserves cardiac function after myocardial infarction. FASEB J 24, 1467-78.

Zhang, F., Tang, Z., Hou, X., Lennartsson, J., Li, Y., Koch, A.W., Scotney, P., Lee, C., Arjunan, P., Dong, L., Kumar, A., Rissanen, T.T., Wang, B., Nagai, N., Fons, P., Fariss, R., Zhang, Y., Wawrousek, E., Tansey, G., Raber, J., Fong, G.H., Ding, H., Greenberg, D.A., Becker, K.G., Herbert, J.M., Nash, A., Yla-Herttuala, S., Cao, Y., Watts, R.J. and Li, X., 2009. VEGF-B is dispensable for blood vessel growth but critical for their survival, and VEGF-B targeting inhibits pathological angiogenesis. Proc Natl Acad Sci U S A 106, 6152-7.

Zhao, T., Zhao, W., Chen, Y., Liu, L., Ahokas, R.A. and Sun, Y., 2012. Differential expression of vascular endothelial growth factor isoforms and receptor subtypes in the infarcted heart. Int J Cardiol.

Zhong, X., Huang, H., Shen, J., Zacchigna, S., Zentilin, L., Giacca, M. and Vinores, S.A., 2011. Vascular endothelial growth factor-B gene transfer exacerbates retinal and choroidal neovascularization and vasopermeability without promoting inflammation. Mol Vis 17, 492-507.

Ziche, M., Maglione, D., Ribatti, D., Morbidelli, L., Lago, C.T., Battisti, M., Paoletti, I., Barra, A., Tucci, M., Parise, G., Vincenti, V., Granger, H.J., Viglietto, G. and Persico, M.G., 1997. Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. Lab Invest 76, 517-31.